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(REV 3-93)

US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY DOCKET NUMBER
2001_0291ATRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. §371U.S. APPLICATION NO.
(if known, add PCT #)
NEW 09/806871International Application No.
PCT/JP99/05456International Filing Date
October 4, 1999Priority Date Claimed
October 5, 1998Title of Invention
METHOD FOR REMOVING N-TERMINAL METHIONINEApplicant(s) For DO/EO/US
Osamu NISHIMURA, Tsuneo ASANO, Masato SUENAGA, Hiroaki OHMAE and Norio OKUTANI


Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. §371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. §371.
3. ☐ This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. §371(c)(2))
 - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☒ A translation of the International Application into English (35 U.S.C. §371(c)(2)). **ATTACHMENT A**
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)):
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☐ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19.
9. ☒ An unexecuted oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). **ATTACHMENT B**
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).

Items 11. to 14. below concern other document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98. **ATTACHMENT C**
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☐ A **FIRST** preliminary amendment.
☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☒ Other items or information: WO 00/20439

THE COMMISSIONER IS AUTHORIZED
TO CHARGE ANY DEFICIENCY IN THE
FEE FOR THIS PAPER TO DEPOSIT
ACCOUNT NO. 23-0975.

U.S. APPLICATION NO. 09/806871 NEW		INTERNATIONAL APPLICATION NO. PCT/IP99/05456		ATTORNEY'S DOCKET NO. 2001 0291A					
15. [X] The following fees are submitted BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00 International Search Report has been prepared by the EPO or JPO \$ 860.00 International preliminary examination fee not paid at USPTO but international search paid to USPTO \$ 710.00 International preliminary examination fee paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) \$ 690.00 International preliminary examination fee paid at USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$ 100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				<table border="1" style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 50%;">CALCULATIONS</th> <th style="width: 50%;">PTO USE ONLY</th> </tr> <tr> <td style="height: 100px;"></td> <td></td> </tr> </table>		CALCULATIONS	PTO USE ONLY		
CALCULATIONS	PTO USE ONLY								
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$					
Claims	Number Filed	Number Extra	Rate						
Total Claims	-20 =		X \$18.00	\$					
Independent Claims	9 - 3 =	6	X \$80.00	\$480.00					
Multiple dependent claim(s) (if applicable)			+ \$270.00	\$					
TOTAL OF ABOVE CALCULATIONS =				\$1,340.00					
<input type="checkbox"/> Small Entity Status is hereby asserted. Above fees are reduced by 1/2.				\$					
SUBTOTAL =				\$1,340.00					
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				+ \$					
TOTAL NATIONAL FEE =				\$1,340.00					
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property +				\$					
TOTAL FEES ENCLOSED =				\$1,340.00					
				Amount to be refunded	\$				
				Amount to be charged	\$				
a. [X] A check in the amount of <u>\$1,340.00</u> to cover the above fees is enclosed. A duplicate copy of this form is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. 23-0975 in the amount of \$_____ to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>23-0975</u> . NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.									
19. CORRESPONDENCE ADDRESS <div style="text-align: center;">  000513 PATENT TRADEMARK OFFICE </div>			By: <u>Warren M. Cheek, Jr.</u> Warren M. Cheek, Jr. Registration No. 33,367 WENDEROTH, LIND & PONACK, L.L.P. 2033 "K" Street, N.W., Suite 800 Washington, D.C. 20006-1021 Phone: (202) 721-8200 Fax: (202) 721-8250 April 5, 2001						

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Rec'd PCT/PTC 07 MAY 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of :
Osamu NISHIMURA et al. : Attn: BOX PCT
Serial No. 09/806,871 : Docket No. 2001_0291A
Filed April 5, 2001 :

METHOD FOR REMOVING N-TERMINAL METHIONINE
[Corresponding to PCT/JP99/05456
Filed October 4, 1999]

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,
Washington, DC 20231

Sir:

Please amend the above-identified application as follows:

IN THE SPECIFICATION

Page 1, immediately after the title, please insert:

This application is a 371 of PCT/JP99/05456 filed October 4, 1999.

REMARKS

The present application has been revised to reflect the 371 status.

Attached hereto is a marked-up version of the changes made to the specification by the current amendment. The attached page is captioned "Version with markings to show changes made".

Favorable action on the merits is solicited.

Respectfully submitted,

Osamu NISHIMURA et al.

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May 7, 2001

SPECIFICATION

Method for Removing N-terminal Methionine

this application is a 371 of PCT/JP99/05456 filed October 4, 1999.

FIELD OF THE INVENTION

5 This invention relates to a method for the
efficient removal, from peptides (including proteins)
or salts thereof which possess an optionally oxidized
N-terminal methionine residue or diketone of said
methionine residue, of the N-terminal methionine
10 residue or the diketone of said methionine residue, in
the presence of acetic acid and sodium formate, formic
acid and sodium formate, or formic acid and sodium
acetate; and to a method for manufacturing peptides or
salts thereof which do not possess an optionally
15 oxidized N-terminal methionine residue or diketone of
said methionine residue.

BACKGROUND ART

When protein is biosynthesized within a cell, its
20 N-terminal is known to start with methionine, which
corresponds to the initiation codon AUG of the mRNA.
However, as this methionine is removed by subsequent
processing, it is usually no longer present in the
completed mature protein molecule.

25 With advancements in recombinant DNA techniques,
it has become possible to produce useful proteins using
microorganisms and/or animal cells, for example
Escherichia coli. There have been instances wherein
protein produced via this type of method was found to
30 retain a residue comprised of the aforementioned
methionine. For example, the retention rate of
methionine was as high as approximately 100% in human
growth hormone expressed in *E. coli* [Nature, 293, 408
(1981)], and 50% in interferon- α [J. Interferon Res., 1,
35 381 (1981)], while in nonglycosylated human
interleukin-2 the presence of a molecular species with

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JC02 Rec'd PCT/PTO 05 APR 2001

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SPECIFICATION

Method for Removing N-terminal Methionine

FIELD OF THE INVENTION

5 This invention relates to a method for the efficient removal, from peptides (including proteins) or salts thereof which possess an optionally oxidized N-terminal methionine residue or diketone of said methionine residue, of the N-terminal methionine
10 residue or the diketone of said methionine residue, in the presence of acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate; and to a method for manufacturing peptides or salts thereof which do not possess an optionally
15 oxidized N-terminal methionine residue or diketone of said methionine residue.

BACKGROUND ART

When protein is biosynthesized within a cell, its
20 N-terminal is known to start with methionine, which corresponds to the initiation codon AUG of the mRNA. However, as this methionine is removed by subsequent processing, it is usually no longer present in the completed mature protein molecule.

25 With advancements in recombinant DNA techniques, it has become possible to produce useful proteins using microorganisms and/or animal cells, for example *Escherichia coli*. There have been instances wherein protein produced via this type of method was found to
30 retain a residue comprised of the aforementioned methionine. For example, the retention rate of methionine was as high as approximately 100% in human growth hormone expressed in *E. coli* [Nature, 293, 408 (1981)], and 50% in interferon- α [J. Interferon Res., 1,
35 381 (1981)], while in nonglycosylated human interleukin-2 the presence of a molecular species with

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methionine retention on the amino-terminal (N-terminal methionine residue) (Met-rIL-2) has been noted in addition to the molecular species rIL-2 which, like naturally-occurring human interleukin-2, is initiated with alanine.

In regards to method for removing N-terminal amino acid chemically, Dixon reported in 1964 that DL-alanylglycine reacts with glyoxylic acid, pyridine, or cupric acetate in a transamination that results in the production of pyruvoylglycine [Biochem. J, 92, 661 (1964)]. He further reported that thiosemicarbazide reacts with compounds to result in amide bond cleavage, producing glycine [Biochem. J, 90, 2C (1964)]. He subsequently applied this reaction to Pseudomonas cytochrome c-551, reporting the removal of N-terminal glutamic acid [Biochem. J, 94, 463 (1965)].

It is reasonable to speculate that the proteins of molecular species that do or do not possess N-terminal methionine might differ from each other in terms of their superstructure, biological activity, and/or stability, and further, that the addition of methionine to the N-terminal could bring about an increase in antigenicity. It is therefore reasonable to surmise, from the perspective of industrial utility, that it is of significance to establish a method for removing the N-terminal methionine that corresponds to the initiation codon.

With the objective of resolving this problem, a method has been proposed wherein methionine is removed via digestion by cyanogen bromide (BrCN) [Science, 198, 1056 (1977)]. However, in addition to presupposing the absence of methionine residue within the desired mature protein, this method, which subjects the protein to harsh chemical reactions, is by no means conducive to satisfactory results.

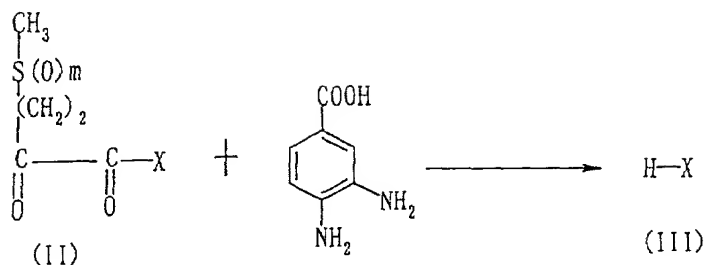
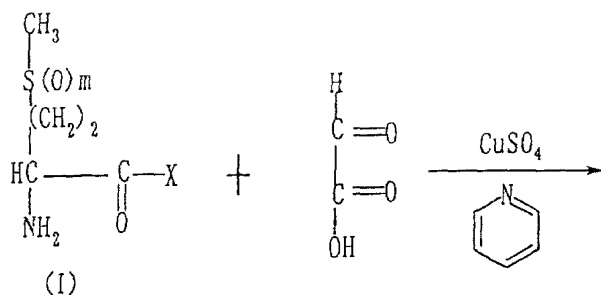
Aside from the method described in Unexamined Kokai Application Heisei 10-72489 (EP-A-812856), chemical methods which remove the N-terminal methionine residue from peptides or proteins which possess an N-terminal methionine residue in a selective and efficient manner regardless of the type of peptide or protein, are not known. This is most likely attributable to the difficulty of identifying a chemical reaction that is capable of removing N-terminal methionine under gentle conditions without altering the final product, i.e. the peptide or protein. In particular, as the removal of excess N-terminal methionine from genetically engineered proteins of relatively large molecular weight, particularly those intended for use as pharmaceutical agents, requires the activity of the protein not to deteriorate upon methionine removal, the reaction usually needs to proceed, without heating, in alkalescent to acescent solutions. As this is extremely restrictive by chemical reaction conditions, the current situation has been such that a favorable set of reaction conditions could not be identified.

DISCLOSURE OF THE INVENTION

The inventors, upon diligent study with the objective of providing, via the exclusive cleavage of the N-terminal methionine residue in peptides (including proteins) manufactured via genetic engineering, a method for the manufacture of peptides with amino acid sequences that mimic naturally occurring sequences, have discovered that it is possible to remove, from peptides which possess a diketone of the methionine residue, the diketone of said methionine residue by having a peptide which possesses an optionally oxidized N-terminal methionine

residue, as represented in Formula (I) of Scheme 1 below, undergo transamination with, for example, glyoxylic acid, which is an α -diketone, or cupric sulfate, which is capable of providing transition metal ions, or pyridine, which is a base (for example, an amine), to yield a peptide which possesses a diketone of the said methionine residue, which, when allowed to react with 3,4 diaminobenzoic acid, which is a base (for example, a diamine), in the presence of acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate, followed by hydrolysis, it is possible to remove the diketone of the methionine residue from the peptide which possesses the diketone of the methionine residue in an unexpectedly efficient manner. Hence the inventors, having identified a method of removing, at an unexpectedly high yields, the optionally oxidized N-terminal methionine residue from peptides which possess methionine residue, to obtain, without bringing about a deterioration in activity, peptides which do not possess optionally oxidized N-terminal methionine residue, further pursued their studies to achieve the completion of the present invention.

(Scheme 1)

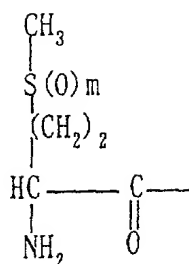


[In formula (I), m represents the integer 0 or 2, while X can be any peptide chain possessing either an amino acid residue or at least 2 amino acids, although from a practical standpoint, an example would be a peptide chain that corresponds to the X of a protein manufactured via genetic engineering. In the specification of the present patent, the term "protein" or "peptide" may refer to a peptide comprised of multiple amino acids or, in the case of a protein, may refer to either a nonglycosylated or glycosylated peptide or protein.]

In the specification of the present patent, within Scheme 1 above,

The compound represented by general formula (I) can be referred to as "a peptide which possesses optionally oxidized N-terminal methionine residue" or "a peptide which possesses methionine residue";

The partial structure within general formula (I) that is represented as

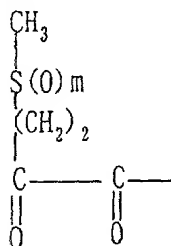


[m within the formula having the same meaning as mentioned above]

- can be referred to as "optionally oxidized methionine residue," "methionine residue," or "methionine";

- The compound represented by general formula (II) can be referred to as "a peptide which possesses the diketone of the optionally oxidized N-terminal methionine residue" or "a peptide which possesses the diketone of the methionine residue";

The partial structure within general formula (II) that is represented as



- [m within the formula having the same meaning as mentioned above]
- can be referred to as "diketone of the optionally oxidized methionine residue" or "diketone of the methionine residue"; and

- The compound represented by general formula (III) can be referred to as "a peptide which does not possess optionally oxidized N-terminal methionine residue" or "a peptide which does not possess the diketone of the optionally oxidized N-terminal methionine residue."

Hence, the present invention relates to

- (1) a method for removing the diketone of N-terminal

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- methionine residue characterized by having a peptide or the salt thereof that possesses the diketone of an optionally oxidized N-terminal methionine residue, react with 3,4-diaminobenzoic acid or the salt thereof
- 5 in the presence of acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate,
- (2) the method described in (1) above wherein the peptide or the salt thereof which possesses the
- 10 diketone of the optionally oxidized N-terminal methionine residue is a peptides or the salt thereof that is obtained by having a peptide or the salt thereof that possesses optionally oxidized N-terminal methionine residue react with an α -diketone,
- 15 (3) the method described in (2) above wherein the peptide that possesses optionally oxidized N-terminal methionine is a peptide that has been manufactured via genetic engineering,
- (4) the method described in (1) above wherein the
- 20 peptide is a (i) growth hormone, (ii) β -cellulin, (iii) interleukin-2, (iv) neutrophin-3, or (v) apelin,
- (5) the method described in (1) above wherein the peptide is a growth hormone,
- (6) the method described in (1) above which is
- 25 characterized by the acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate, being used as a buffering solution of approximately 0.1 to 8 mol/L with a pH of approximately 2 to 9,
- 30 (7) a method for removing the diketone of the N-terminal methionine residue characterized by having a peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue react with 3,4-diaminobenzoic acid
- 35 or the salt thereof in the presence of acetic acid and

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sodium formate,

(8) a method of manufacturing a peptide or the salt thereof which does not possess the optionally oxidized N-terminal methionine residue characterized by having a

5 peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue react with 3,4-diaminobenzoic acid or the salt thereof in the presence of acetic acid or sodium formate, or formic acid and sodium formate, or
10 formic acid and sodium acetate,

(9) a method of manufacture described in (8) above wherein the peptide or the salt thereof that possesses the diketone of the optionally oxidized N-terminal methionine residue is a peptide or the salt thereof
15 that is obtained by having a peptide or the salt thereof which possesses optionally oxidized N-terminal methionine residue react with an α -diketone,

(10) a method of manufacture described in (8) above characterized by the acetic acid and sodium formate, or
20 formic acid and sodium formate, or formic acid and sodium acetate, being used as a buffering solution of approximately 0.1 to 8 mol/L with a pH of approximately 2 to 9,

(11) a method of manufacturing a peptide or the salt
25 thereof that does not possess N-terminal methionine residue characterized by having a peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue react with 3,4-diaminobenzoic acid or the salt thereof in the presence
30 of acetic acid and sodium formate,

(12) a method of manufacturing human growth hormone or the salt thereof which does not possess N-terminal methionine residue characterized by having the human growth hormone or the salt thereof that is manufactured
35 via genetic engineering which possesses optionally

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oxidized N-terminal methionine residue react with glyoxylic acid or the salt thereof in the presence of cupric sulfate and pyridine, and subsequently with 3,4-diaminobenzoic acid or the salt thereof in the presence of acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate, (13) the use of (i) acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or the salt thereof, for the purpose of removing the N-terminal methionine residue from a peptide or the salt thereof which possesses optionally oxidized N-terminal methionine residue, (14) the use of (i) acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or the salt thereof, for the purpose of removing the diketone of the methionine residue from a peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue, (15) the use of (i) acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or the salt thereof, for the purpose of manufacturing a peptide or the salt thereof which does not possess optionally oxidized N-terminal methionine residue from a peptide or the salt thereof which possesses optionally oxidized N-terminal methionine residue, (16) the use of (i) acetic acid and sodium formate, or formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or the salt thereof, for the purpose of manufacturing a peptide or the salt thereof which does not possess the diketone of the optionally oxidized N-terminal methionine residue from a peptide or the salt thereof

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which possesses the diketone of the optionally oxidized N-terminal methionine residue.

BRIEF DESCRIPTION OF THE DRAWINGS

5 Figure 1 is an illustration of the results of electrophoresis obtained in Example 4 a). Lane 1 represents the molecular weight marker, while Lane 2 represents the purified hGH.

10 Figure 2 is an illustration of the results of electrophoresis obtained in Example 16 a). Lane 1 represents the molecular weight marker, while Lane 2 represents the BTC obtained in Example 15.

15 Figure 3 is an illustration of the results of electrophoresis obtained in Example 18 a). Lane 1 represents the molecular weight marker, while Lane 2 represents the IL-2 obtained in Example 17.

Figure 4 is an illustration of the DNA fragment used in Example 26.

20 Figure 5 is an illustration of the schematic diagram for manufacturing the double chain human apelin-36 obtained in Example 26.

Figure 6 is an illustration of the structural diagram of plasmid pTB 960-13 obtained in Example 27.

25 BEST MODE OF EMBODIMENT OF THE INVENTION

In the specification of the present patent, the optionally oxidized methionine residue indicates the methionine residue or its sulfoxide, while the sulfoxide of the methionine residue can include 30 methionine sulfoxide or methionine sulfone.

While a peptide which possesses optionally oxidized N-terminal methionine residue can indicate a peptide which is represented by the formula $\text{CH}_3\text{-S(O)}_m\text{-(CH}_2)_2\text{-CO-X}$ [where m indicates the integer 0 or 2, and 35 X indicates the amino acid residue or the peptide

chain], such peptide can also be in the form of a salt, of which any kind is acceptable insofar as such salt does not inhibit the reaction of the present invention, but which preferably is a pharmaceutically acceptable salt including a salt with an inorganic acid such as hydrochloric acid, hydrobromic acid, nitric acid, sulfuric acid, or phosphoric acid, a salt with an organic acid such as acetic acid, pthalic acid, fumaric acid, tartaric acid, maleic acid, citric acid, succinic acid, methanesulfonic acid, or para-toluenesulfonic acid, an alkaline metal salt such as a sodium salt or a potassium salt, an alkaline earth metal salt such as calcium salt, or an ammonium salt.

A peptide which possesses optionally oxidized N-terminal methionine residue is preferably a peptide manufactured by genetic engineering which possesses optionally oxidized N-terminal methionine residue.

In the formula above, 0 is preferable as the m, while a peptide chain with at least two amino acids is preferable as X.

The peptide in the present invention can either be a "peptide," with fewer than 50 amino acids, or a "protein," with at least 50 amino acids.

In this manner, while the term "peptide" in the specification of the present patent includes not only a molecule with fewer than 50 amino acids but also a molecule with at least 50 amino acids, it is preferable to use a molecule with at least 50 amino acids ("a protein").

A preferable peptide is a peptide comprised of 2 to 1,000 amino acids, of which a particularly preferable peptide is one comprised of 15 to 500 amino acids, of which a specific example is a protein such as a growth hormone (GH) [for example, human growth hormone (hGH) (e.g. 20K-hGH and 22K-hGH)], β -cellulin

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thymopoietin, thymosin, Factor XIII, Factor VIII, prourokinase, SOD, Factor VIIa, antithrombin, or apelin, or the mutein thereof (in which at least one amino acid is replaced, deleted, or added in a wild type protein, showing a biological or immunological activity equal to or greater than that of the wild type protein), or a known or new peptide manufactured via a method such as chemical synthesis, of which a particularly preferable peptide for use is a peptide (which can also be a protein) manufactured via genetic engineering, in particular a growth hormone manufactured via genetic engineering [for example, human growth hormone (hGH) (e.g. 20K-hGH and 22K-hGH)], neurotrophin-3, β -cellulin, parathyroid hormone, interleukin-2, apelin, or the mutein thereof, in particular a growth hormone [for example, human growth hormone (hGH) (e.g. 20K-hGH and 22K-hGH)]. The aforementioned apelin can be, for example, as described in Biochem. Biophys. Res. Commun., 251, 471-476 (1998), human apelin-36, human apelin-13, or a peptide in which the N-terminal amino acid (Gln) of apelin-13 has been converted to pyroglutamic acid; any type of peptide is acceptable insofar as such peptide has ligand activity toward APJ (O'Dowd, B.F., et al, Gene, 436, 355-359 (1993)), a specific example of which is, as described in Patent Application Heisei 10-271654, "a polypeptide which has the capability to bind with a receptor protein which has an identical, or practically identical, amino acid sequence to the amino acid sequence represented by sequence number: 3."

While the aforementioned peptide (which can also be a wild type protein) mentioned above can be derived from any animal species, a human derived peptide (which can also be a protein) is preferable for practical use.

The aforementioned peptide can be refolded (activated, regenerated) prior to or following being

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subjected to the process of removing the optionally oxidized N-terminal methionine (Met) residue or the diketone of said methionine residue.

5 In the specification of the present patent, a peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue indicates a compound or the salt thereof which is represented by the formula $\text{CH}_3\text{-S(O)}_m\text{-(CH}_2\text{)}_2\text{-CO-CO-X}$ [where m indicates the integer 0 or 2, and X indicates the amino acid residue or the peptide chain]. The peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue can be obtained via a variety of reactions, such as a chemical reaction or an enzyme reactions. For example, a method of chemical reaction might be one in which a peptide or the salt thereof which possesses the diketone of the optionally oxidized N-terminal methionine residue is obtained via transamination, in which a peptide or the salt thereof which possesses optionally oxidized N-terminal methionine residue is made to react with an α -diketone (Unexamined Kokai Application Heisei 10-72489 (EP-A-812856)).

25 In the specification of the present patent, the α -diketone can be of any kind insofar as such α -diketone allows the transamination of the above mentioned peptide or the salt thereof to proceed, an examples of which is a compound represented by the formula $\text{R}^1\text{-CO-CO-R}^2$ [where R^1 indicates a low alkyl or phenyl group optionally substituted with hydrogen or a carboxyl group (preferably hydrogen or methyl, more preferably hydrogen), and R^2 indicates an amino group optionally substituted with a hydroxyl group, low alkoxy group, or low alkyl (preferably a hydroxyl group)], or the salt thereof.

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In the above formula, an example of the low alkyl group indicated by R^1 is an alkyl group with 1 to 6 carbons, such as methyl, ethyl, propyl, i-propyl, butyl, i-butyl, sec-butyl, or t-butyl, while an example of the low alkoxy group indicated by R^2 is an alkoxy group with 1 to 6 carbons, such as methoxy, ethoxy, propoxy, i-propoxy, butoxy, i-butoxy, sec-butoxy, or t-butoxy. An example of the amino group optionally substituted with low alkyl indicated by R^2 is an amino group that can have one or two low alkyl groups indicated by aforementioned R^1 . An example of a salt is a salt that is similar to a salt of the aforementioned peptide which possesses optionally oxidized N-terminal methionine residue.

Some specific examples of an α -diketone would be glyoxylic acid, pyruvic acid, oxalacetic acid, phenylglyoxylic acid, and 2-oxoglutaric acid, of which glyoxylic acid is particularly preferable for use.

The α -diketone can be in the form of a salt, of which an example is an alkaline metal salt such as a sodium salt or a potassium salt, or an inorganic salt such as a hydrochloride.

The transamination of a peptide or the salt thereof which possesses an optionally oxidized N-terminal methionine residue with an α -diketone is usually preferably conducted with approximately 1 to 10,000 moles (preferably 2,000 to 4,000 moles) of α -diketone for 1 mol of peptide or the salt thereof, at approximately 0 to 70°C (preferably approximately 20 to 40°C) for approximately 10 minutes to 5 hours (preferably approximately 20 minutes to 2 hours). Insofar as it does not inhibit the above-mentioned transamination, any buffer solution can be used (e.g. phosphate buffer solution, acetate buffer solution, or citrate buffer solution), although acetate buffer

solution is particularly preferably for use. The pH for the reaction is best adjusted to approximately 2 to 9, in particular approximately 4 to 7, above all approximately 5 to 6, for the reaction to proceed.

5 To facilitate the said transamination, it is preferable to have the α -diketone react in the presence of a transition metal ion, of which the use of approximately 0.001 to 0.1 moles (preferably 0.01 to 0.05 moles) of transition metal ion to 1 mole of α -
10 diketone is usually preferable. Examples of transition metal ions that can be used include copper (Cu^+ , Cu^{2+}), cobalt (Co^{2+} , Co^{3+}), nickel (Ni^{2+} , Ni^{3+}), iron (Fe^{2+} , Fe^{3+}), zinc (Zn^{2+}), aluminum (Al^{3+}), manganese (e.g. Mn^{2+}), gallium (Ga^{3+}), indium (In^{3+}), magnesium (Mg^{2+}), and
15 calcium (Ca^{2+}), out of which such ions as copper or cobalt, in particular copper (Cu^{2+}), is particularly preferable for use. The transition metal ion can be added to the reaction solvent, usually in the form of a salt of an inorganic acid such as sulfuric acid, nitric
20 acid, hydrochloric acid, or perchloric acid, or of an organic acid such as acetic acid, oxalic acid, citric acid, or carbonic acid, of which cupric sulfate or cupric acetate, particularly cupric sulfate, is preferable for use.

25 Additionally, it is preferable to have the α -diketone react in the presence of a base, wherein 0.1 to 20 moles (preferably 0.5 to 10 moles) of base for 1 mole of α -diketone is usually preferable for use. Some examples of a base include an alkylamine such as
30 triethylamine, tributylamine, or an aromatic amine such as N,N-dimethylaniline, pyridine, lutidine, collidine, 4-(dimethylamino)pyridine, or imidazole, of which pyridine is particularly preferable for use.

35 Additionally, for the purposes of preventing the precipitation during transamination of the peptide or

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the salt thereof which possesses an optionally oxidized N-terminal methionine residue, or the peptide or the salt thereof which possesses the diketone of the methionine residue obtained via transamination of the peptide or the salt thereof which possesses an optionally oxidized N-terminal methionine residue, it is preferable to add urea to the transamination buffer solution in accordance with the type of said peptide, peptide which possesses the diketone of the methionine residue, or the salt thereof. For example, when using hGH, it is preferable to add urea to the buffer solution so that the concentration is approximately 1 to 8 M, more preferably approximately 3 to 6 M.

Further, in the aforementioned transamination, it is preferable to have the α -diketone react in the presence of a transition metal ion and a base, wherein for practical purposes, the transamination is made to proceed with the addition of a mixed solution containing the three components of transition metal ion, base, and α -diketone (for example, cupric sulfate, pyridine, and glyoxylic acid) to the aqueous solution containing the peptide or the salt thereof which possesses optionally oxidized N-terminal methionine residue.

While the compound or the salt thereof that is obtained by said transamination and is represented by the formula $\text{CH}_3\text{-S(O)}_m\text{-(CH}_2)_2\text{-CO-CO-X}$ [where m indicates the integer 0 or 2, and X indicates the amino acid residue or peptide chain] can be isolated from the reaction solution and purified via known procedures for peptide or protein purification, for example, extraction, salting out, distribution, recrystallization, or chromatography, said compound or the salt thereof can also be subjected, as is, to the following hydrolysis reaction.

The peptide or the salt thereof obtained via the transamination which possesses the diketone of the methionine can usually be converted to an amino acid, peptide, or the salt thereof which does not possess optionally oxidized N-terminal methionine residue or diketone of said methionine residue.

While the base used in the hydrolysis reaction can be, for example, an alkylamine such as cysteamine, triethylamine, or tributylamine, or a salt thereof, an aromatic amine such as N,N-dimethylaniline, pyridine, lutidine, collidine, 4-(dimethylamino)pyridine, or imidazole, or a salt thereof, a diamine (preferably an aromatic diamine, in particular 3,4-diaminobenzoic acid or an n-alkyl substitution thereof (for example, n-methyl-1,2-phenylenediamine, n-ethyl-1,2-phenylenediamine, or n-isopropyl-1,2-phenylenediamine) such as o-phenylenediamine, tolylene-3,4-diamine, 3,4-diaminobenzoic acid or an n-alkyl substitution thereof (for example, n-methyl-1,2-phenylenediamine, n-ethyl-1,2-phenylenediamine, or n-isopropyl-1,2-phenylenediamine), 2,3-diaminophenol, or 4-chloro-o-phenylenediamine, or a salt thereof, a thiosemicarbazide such as thiosemicarbazide, acetone thiosemicarbazide, or phenyl thiosemicarbazide, a selenosemicarbazide such as selenosemicarbazide or acetone selenosemicarbazide or a salt thereof, an amine is preferable, in particular a diamine or thiosemicarbazide or a salt thereof, and especially 3,4-diaminobenzoic acid or a salt thereof.

An example of a base that can be used in the hydrolysis reaction can be a base which is similar to a salt of the peptide which possesses optionally oxidized N-terminal methionine residue mentioned above.

The amount of base is usually approximately 1 to 10,000 moles for 1 mole of the peptide which possesses

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the diketone of the methionine residue, or the salt thereof, preferably approximately 200 to 3,000 moles, and more preferably, approximately 500 to 3,000 moles. The hydrolysis reaction is usually made to proceed at approximately 0 to 70°C (preferably approximately 20 to 40°C) for approximately 1 hour to 7 days (preferably approximately 10 hours to 5 days). It is preferable to use a buffer solution as the solvent in the reaction, an example of such a buffer solution being a formic acid buffer (for example, acetic acid-sodium formate, formic acid-sodium formate, or formic acid-sodium acetate). While any buffer solution can be used insofar as such buffer solution does not inhibit the aforementioned hydrolysis reaction, an acetic acid-sodium formate, formic acid-sodium formate, or formic acid-sodium acetate buffer solution is particularly preferable. The pH for the reaction is best adjusted to approximately 2 to 9, in particular approximately 3 to 7, above all approximately 4 to 6, for the reaction to proceed. The amount of buffer solution to use is preferably approximately 0.1 to 8 mol/L, and more preferably approximately 0.5 to 6 mol/L.

In order to prevent the precipitation of the amino acid, peptide, or salt thereof which does not possess optionally oxidized N-terminal methionine obtained via hydrolysis of a peptide or salt thereof which possesses a diketone of optionally oxidized N-terminal methionine, it is preferable to add urea to the buffer solution for hydrolysis in accordance with the type of said peptide which possesses a diketone of optionally oxidized N-terminal methionine and the type of said amino acid, peptide, or salt thereof which does not possess methionine residue. For example, when using hGH, it is preferable to add urea to the buffer solution so that the concentration is approximately 1 to 6 M, more

preferably approximately 2 to 5 M.

While the amino acid, peptide, or salt thereof obtained in this way can be isolated from the reaction solution and purified via known methods of purification, for example extraction, salting out, distribution, recrystallization, or chromatography, a preferable example is purification via ion exchange chromatography using SP-Sepharose (Pharmacia Biotech) or DEAE-5PW (Tosoh Corporation).

As the peptide manufactured in the present invention is obtained in a form that does not possess methionine on its N-terminal, and that furthermore has an amino acid sequence identical to that of the biologically active target peptide (for example, a biologically active native polypeptide), such peptide possesses similar activity to that of the target peptide (for example, a native polypeptide), has a low toxicity, and can be used safely as a pharmaceutical or diagnostic agent.

The present invention makes possible the specific removal of the methionine residue or the diketone of said methionine residue from a peptide which possesses an optionally oxidized N-terminal methionine residue or a diketone of said methionine residue.

Where abbreviations have been used to indicate amino acids etc. in the patent specification or drawing of the present invention, such abbreviations are in accordance with the IUPAC-IUB Commission on Biochemical Nomenclature or in common use in the relevant field, as shown in the examples below. When an amino acid shows optical isomerism, the L-form is indicated unless explicitly stated otherwise.

SDS: sodium dodecyl sulfate

Gly: glycine

Ala: alanine
Val: valine
Leu: leucine
Ile: isoleucine
5 Ser: serine
Thr: threonine
Cys: cysteine
Met: methionine
Glu: glutamic acid
10 Gln: glutamine
Asp: aspartic acid
Asn: asparagine
Lys: lysine
Arg: arginine
15 His: histidine
Phe: phenylalanine
Tyr: tyrosine
Trp: tryptophan
Pro: proline
20 Asx: Asp + Asn
Glx: Glu + Gln

While the present invention is more specifically described by the references and examples below, the present invention is not limited by these references and examples.

Reference 1 (Construction of human growth hormone (hGH) expression vector using T7 promoter)

30 The structural gene for hGH was recovered from a human pituitary cDNA library (Quick-Clone, CLONTECH Laboratories, Inc.) via PCR amplification using a primer with an Nde I cleavage site immediately upstream of the initiation codon of the structural gene, and a
35 primer with a Bam HI cleavage site immediately

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downstream of the termination codon of the structural gene. The hGH enzyme gene thus obtained with restriction enzyme recognition sites added at both ends was ligated into the T-cloning site of pT7 Blue (DNA Ligation Kit Ver. 2, Takara Shuzo Co., Ltd.) to create pT7HGH-Na. This was introduced into *E. coli* JM109, whereupon transformants were selected by ampicillin resistance and β -galactosidase activity.

Meanwhile, the expression vector was constructed in the following manner. pBR322 was cut with Nde I, whereupon the ends were blunted with T4 DNA polymerase (DNA Blunting Kit, Takara Shuzo Co., Ltd.), then religated, to yield pBRdesNde with the Nde I recognition site deleted. pET3c was cut with Bgl II-Eco RV, whereupon fragments of approximately 0.26 kbp were recovered, then the ends blunted with T4 DNA polymerase, and subsequently ligated into Sca I fragments of pBRdesNde, to yield pBR/T7 desNde. Additionally, by site-directed mutagenesis (Quick Change, Stratagene) pBR322desBam, lacking the Bam HI recognition site of pBR322 was prepared. The Sph I-Eco RV fragment of pBR322desBam was ligated to the Sph I-Eco RV fragment of pBR/T7 desNde to yield the tetracycline resistant expression vector pTC. Vectors in which the tetracycline resistant gene and the T7 promoter were in the opposite direction were designated pTC1, while vectors in which said gene and promoter were in the same direction were designated pTC2.

pT7HGH-Na was cut with Nde I and Bam HI to harvest the hGH structural gene, which was then ligated into the Nde I-Bam HI fragment of pTC1, then subsequently introduced into *E. coli* JM109, whereupon transformants were selected by ampicillin resistance, from which strain plasmids were recovered, and designated expression plasmid pTCHGH-Na.

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E. coli MM294 was lysogenized by a recombinant lambda-phage containing the T7 RNA polymerase gene (Studier, supra). Subsequently, the hGH expression vector pTCHGH-Na was introduced into this *E. coli* MM294(DE3), to yield *E. coli* MM294(DE3)/pTCHGH-Na. The base sequence of hGH was confirmed by DNA sequencer ABI Prism 377A at the time pT7HGH-Na was created.

Reference 2 (Expression of hGH which possesses methionine residue (Met-hGH) in *E. coli*)

The transformed cells obtained in Reference 1 were subjected to 16 hours of shake culture at 30°C, within a 2-liter flask containing 1 liter of LB medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride) containing 5 mg/L tetracycline. The culture solution obtained was transplanted to a 50 liter fermenter with 20 liters of LB medium containing 0.02% Newpol LB-625 (antifoaming agent, Sanyo Chemical Industries Ltd.) and 5 mg/L tetracycline, to undergo 6 hours of aeration/agitation at 37°C. This culture solution was transplanted to a 500 liter fermenter containing 360 liters of fermentation medium (1.68% dibasic sodium phosphate, 0.3% monobasic potassium phosphate, 0.1% ammonium chloride, 0.05% sodium chloride, 0.05% magnesium sulfate, 0.05% Newpol LB-625, 0.0005% thiamine hydrochloride, 1.5% glucose, 3.0% Hy-Case Amino, 1.0% yeast extract), to start aeration/agitation at 37°C. When the turbidity of the culture reached approximately 1,200 Klett units, 17.85 mg/L of isopropyl- β -D-thiogalactopyranoside (IPTQ) was added, with a further 24 liters of 30% glucose added while culturing was continued, whereupon 5 hours later the culture was subjected to centrifugation to obtain approximately 12.3 kg of wet cells, which were then frozen for storage at -80°C.

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The aforementioned transformed *E. coli* (MM294(DE3), pTCHGH-Na) was deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry on December 10, 1997, under accession number FERM P-16546, which was then transferred to an international depository on September 24, 1999, under accession number FERM BP-6888. The aforementioned transformed *E. coli* (MM294(DE3), pTCHGH-Na) was deposited with the Institution for Fermentation, Osaka (IFO) on October 16, 1997, under accession number IFO 16124.

Example 1 (Activation of Met-hGH)

To 2 kg of the cells obtained in Reference 2, 6 liters of 50 mM Tris/HCl and 8M guanidine hydrochloride solution (pH 8.0) was added to dissolve the cells, which were then subjected to sonication using an ultrasonic cell disruptor (Sonifier 450, Branson Ultrasonics Corporation), then subsequently subjected to centrifugation (10,000 rpm for 120 minutes). To 6 liters of the supernatant obtained, 18 liters of 50 mM Tris/HCl, 0.28 mM GSSG, 1.4 mM GSH, and 0.7 M arginine (pH 8.0) was added, the pH adjusted to 8.0, and activation conducted for 4 days at 4°C.

Example 2 (Purification of Met-hGH)

The regenerant which underwent activation in Example 1 was desalted and concentrated in a Pellicon cassette system (PTGC membrane, Millipore Corporation) with the addition of 20 mM Tris/HCl and 2.5 M urea (pH 8.0), until the electric conductivity was no greater than 10 mS, whereupon 50 mM phosphate buffer solution (pH 6.0) was added to 5 liters of the concentrate obtained to dilute it to 50 liters, which was then

allowed to stand overnight at 4°C. Subsequently, continuous centrifugation was conducted (JCF-Z Rotor, Beckman Inc.), whereupon 10 M sodium hydroxide was added to 50 liters of supernatant obtained to adjust the pH to 7.12, whereupon it was concentrated in a Pellicon cassette system (PTGC membrane, Millipore Corporation), and following replacement of buffer solution with 20 mM Tris/HCl (pH 8.0), was centrifuged (10,000 rpm for 30 minutes) to obtain supernatant. Subsequently, this supernatant was allowed to adsorb to DEAE-Toyopearl 650 M column (20 cmφ × 84 cm, Tosoh Corporation) equilibrated with 20 mM Tris/HCl (pH 8.0), underwent a thorough wash, then eluted with 20 mM Tris/HCl containing 50 mM sodium chloride (pH 8.0), to obtain 95 liters of eluate as Met-hGH fractions. Further, this eluate was concentrated and desalted in a Pellicon cassette system (PTGC membrane, Millipore Corporation), then the buffer solution replaced with 20 mM Tris/HCl and 6 M urea (pH 8.0), to obtain 12.21 grams of Met-hGH.

Example 3 (Removal of N-terminal methionine residue (N-terminal Met))

To 1,800 milliliters of the Met-hGH solution obtained in Example 2, 450 milliliters of 2.5 M glyoxylic acid, 40 mM cupric sulfate, and 50% pyridine solution was added and stirred well, then allowed to react for 60 minutes at 25°C. This was subsequently allowed to apply to a Sephadex G-25 column (11.3 cmφ × 125 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 4.0 M urea (pH 8.0) at a flow rate of 3 liters/h, and developed using the same buffer solution as was used for equilibration, to obtain 4.2 liters of eluate as hGH fractions possessing a diketone of the methionine residue. This eluate was added into 20.8

liters of 1.2 M acetic acid, 2.4 M sodium formate, 3.6 M urea solution, and 48 mM 3,4-diaminobenzoic acid solution while stirring well, then allowed to react for 3 days at 30°C while stirring slowly. Following the reaction, this solution was concentrated to 14 liters in a Pellicon cassette system (PTGC membrane, Millipore Corporation), then divided into 2 runs of 7 liters each to be applied at a flow rate of 6 liters/h to a Sephadex G-25 column (25.2 cmφ × 50 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 4.0 M urea (pH 8.0), to collect 20 liters of hGH fraction. Subsequently, via high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.) this solution was subjected to flow adsorption in a DEAE-5PW column (21 cm × 30 cm, Tosoh Corporation), then was eluted at a flow rate of 320 milliliters/minute for 70 minutes in a pH gradient of 70 to 85% B generated with A = 50 mM Tris/HCl + 2.5 M urea (pH 8.0) and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid] + 2.5 M urea (pH 4.0), to obtain 5.84 liters of hGH fractions. To this hGH fraction, 16 milliliters of 10 M NaOH solution was added to adjust the pH to 7.1, whereupon high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.) was conducted in 8 separate batches. Upon allowing the designated amount of the concentrate to flow through and adsorb to a POROS 20R1 column (5 cm × 60 cm, Nihon PerSeptive Ltd.), elution was conducted at a flow rate of 50 milliliters/minute for 150 minutes in a pH gradient of 50 to 85% B generated with A = 25% n-propanol + 75% 50 mM Tris/HCl (pH 8.5) and B = 35% n-propanol + 65% 50 mM Tris/HCl (pH 8.5), to obtain 34.7 liters of eluate as hGH fraction. Distilled water was added to this eluate to dilute it to 200 liters, then following concentration to 5 liters in a Pellicon cassette system (PTGC

membrane, Millipore Corporation), this solution was made to flow through and adsorb to a DEAE-5PW column (10.8 cm × 20 cm, Tosoh Corporation) in 3 separate batches by high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.), then eluted at a flow rate of 80 milliliters/minute for 70 minutes in a pH gradient of 70 to 85% generated with A = 50 mM Tris/HCl + 2.5 M urea (pH 8.0) and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid] + 2.5 M urea (pH 4.0), to obtain 1,616 milliliters of hGH fraction. To this hGH fraction, 2 milliliters of 10 M NaOH solution was added to adjust the pH to 7.1, followed by concentration with an ultrafilter (Omega membrane, Fuji Filter Mfg. Co., Ltd.) to obtain 0.4 liters of concentrate. This concentrate was made to flow through a Sephacryl S-100 column (11.3 cmφ × 50 cm, Pharmacia Biotech, Inc.) at a flow rate of 2 liters/h, and developed to obtain hGH fraction. Further, this solution was filtered through a Millipack 60 (Millipore Corporation) to obtain 2,391 milliliters of hGH solution (4,638 milligrams of hGH).

Example 4 (Characterization of hGH)

(a) Analysis using SDS polyacrylamide gel electrophoresis

To the hGH obtained in Example 3, an equal volume of sample buffer containing 100 mM DTT [Laemmli, Nature, 227, 680 (1979)] was added and stirred well, followed by heating for 2 minutes at 95°C, whereupon electrophoresis was performed using Multigel 10/20 (Daiichi Pure Chemicals Co., Ltd.). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, whereupon a single band was observed at approximately 22 KDa, thereby confirming that the purified hGH was monomeric (Figure 1).

(b) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Perkin Elmer Applied Biosystems Model 477A). The hGH N-terminal amino acid sequence thus obtained matched the hGH N-terminal amino acid sequence that was deduced from the cDNA base sequence (Table 1).

10 Table 1

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid deduced from hGH base sequence
1	Phe (848)	Phe
2	Pro (520)	Pro
3	Thr (403)	Thr
4	Ile (620)	Ile
5	Pro (401)	Pro
6	Leu (429)	Leu
7	Ser (92)	Ser
8	Arg (262)	Arg
9	Leu (376)	Leu
10	Phe (283)	Phe
11	Asp (182)	Asp
12	Asn (175)	Asn
13	Ala (175)	Ala
14	Met (194)	Met
15	Leu (261)	Leu
16	Arg (181)	Arg
17	Ala (144)	Ala
18	His (80)	His
19	Arg (152)	Arg
20	Leu (200)	Leu
21	His (71)	His

1) Phenylthiohydantoin

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Analysis was performed using 1 nmol.

(c) Amino acid composition analysis

The amino acid composition was determined using an amino acid analyzer (L-8500A, Hitachi, Ltd.). The hGH amino acid composition thus obtained matched the amino acid composition deduced from the cDNA base sequence (Table 2).

Table 2

Amino acid	Number of residues per mole	Value deduced from hGH base sequence
Asx	19.8	20
Thr ¹⁾	9.7	10
Ser ¹⁾	16.1	18
Glx	27.0	27
Pro	7.7	8
Gly	8.0	8
Ala	6.9	7
Cys ²⁾	N.D.	4
Val	6.8	7
Met	2.9	3
Ile	7.4	8
Leu	26.6	26
Tyr	7.9	8
Phe	12.4	13
His	3.0	3
Lys	8.7	9
Arg	10.7	11
Trp	0.9	1

Acid hydrolysis (mean value of 24-hours and 48-hour hydrolysis, at 110°C, with 6N HCl - 4% thioglycolic acid)

1) Value extrapolated at 0 hours.

2) Not detected.

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Analysis was performed using approximately 10 μ g.

(d) C-terminal amino acid analysis

The C-terminal amino acid was determined using an amino acid analyzer (L-8500A, Hitachi, Ltd.). The hGH C-terminal amino acid thus obtained matched the C-terminal amino acid deduced from the cDNA base sequence (Table 3).

Table 3

C-terminal amino acid	Recovery rate (%)
Phe	94

Phase hydrazinolysis (at 100°C for 6 hours)
Analysis performed using 20 nmol.

Example 5 (Measurement of hGH activity)

The growth promoting activity of the purified hGH obtained in Example 3 on Nb2 cells [Journal of Clinical Endocrinology and Metabolism, Vol. 51, p. 1058 (1980)] was equivalent to that of the reference material (Chemicon International, Inc., Temecula, California, USA).

Example 6 (Removal of N-terminal Met)

20 mM Tris/HCl and 4.0 M urea (pH 8.0) was added to 0.4 milliliters of hGH fraction possessing diketone of the methionine residue obtained in Example 3, to dilute it to 2 milliliters. An equal volume of 4 M acetic acid, 4 M sodium acetate, 6 M urea solution, and 80 mM N-methyl-1,2-phenylenediamine solution was added to said solution, stirred well, and was allowed to react for 20 hours at 30°C. Following the reaction, the solution was applied to a Sephadex G-25 column (1 cm ϕ \times 30 cm, Pharmacia Corporation) equilibrated with

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20 mM Tris/HCl and 4.0 M urea (pH 8.0) at a flow rate of 60 milliliters/h, to collect 10 milliliters of hGH fraction. Subsequently, by high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.), this solution was applied to a DEAE-5PW column (2.15 cm x 15 cm, Tosoh Corporation) and made to flow through and adsorb, followed by elution at a flow rate of 7.5 milliliters/minute for 70 minutes in a pH gradient of 70 to 85% B generated with A = 50 mM Tris/HCl + 2.5 M urea (pH 8.0) and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid] + 2.5 M urea (pH 4.0), to obtain hGH.

Example 7 (Removal of N-terminal Met)

20 mM Tris/HCl and 4.0 M urea (pH 8.0) was added to 0.4 milliliters of hGH fraction possessing diketone of the methionine residue obtained in Example 3, to dilute it to 2 milliliters. An equal volume of 2 M acetic acid, 4 M sodium formate, 6 M urea solution, and 80 mM N-methyl-1,2-phenylenediamine solution was added to this diluent, stirred well, and allowed to react for 20 hours at 30°C. After the reaction, the solution was applied to a Sephadex G-25 column (1 cmφ x 30 cm, Pharmacia Corporation) equilibrated with 20 mM Tris/HCl and 4.0 M urea (pH 8.0) at a flow rate of 60 milliliters/h, to collect 10 milliliters of hGH fraction. Subsequently, in high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.), this solution was applied to a DEAE-5PW column (2.15 cm x 15 cm, Tosoh Corporation) and made to flow through and adsorb, followed by elution at a flow rate of 7.5 milliliters/minute for 70 minutes in a pH gradient of 70 to 85% B generated with A = 50 mM Tris/HCl + 2.5 M urea (pH 8.0) and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid] + 2.5 M urea (pH 4.0),

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to obtain hGH.

Example 8 (Removal of N-terminal Met)

To 1.8 ml of the Met-hGH solution obtained in
5 Example 2, 0.45 ml of 2.5 M glyoxylic acid, 40 mM
cupric sulfate, and 50% pyridine solution was added and,
after stirring well, was allowed to react for 60
minutes at 25°C. This was subsequently applied at a
flow rate of 100 ml/h to a Sephadex G-25 column (1.5
10 cmφ × 30 cm, Pharmacia Biotech, Inc.) equilibrated with
20 mM Tris/HCl and 4.0 M urea (pH 8.0), and developed
using the same buffer solution as was used for
equilibration, to obtain 10 ml of eluate as hGH
fractions possessing diketone of the methionine residue.
15 This eluate was added into 49.5 ml of 1.2 M acetic acid,
2.4 M sodium formate, 3.6 M urea solution, and 48 mM
3,4-diaminobenzoic acid solution while stirring well,
then allowed to react for 24 hours at 37°C while
stirring slowly. Following the reaction, it was then
20 applied at a flow rate of 500 ml/h to a Sephadex G-25
column (4.6 cmφ × 60 cm, Pharmacia Biotech, Inc.)
equilibrated with 20 mM Tris/HCl and 4.0 M urea (pH
8.0), to collect 150 ml of hGH fractions. Subsequently,
in high speed liquid chromatography (Gilson HPLC system,
25 Gilson Company, Inc.) this solution was made to flow
through and adsorb to a DEAE-5PW column (2.15 cm × 15
cm, Tosoh Corporation), followed by elution at a flow
rate of 7.5 ml/minute for 70 minutes in a pH gradient
of 70 to 85% B generated with A = 50 mM Tris/HCl + 2.5
30 M urea (pH 8.0) and B = 50 mM MES [2-(N-morpholino)-
ethane sulfonic acid] + 2.5 M urea (pH 4.0), to obtain
6.7 mg of hGH fractions.

Example 9 (Removal of N-terminal Met)

35 0.45 ml of 2.5 M glyoxylic acid, 40 mM cupric

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sulfate, and 50% pyridine solution was added to 1.8 ml of the Met-hGH solution obtained in Example 2, stirred well, and was allowed to react for 60 minutes at 25°C. This was subsequently applied at a flow rate of 100 ml/h to a Sephadex G-25 column (1.5 cm ϕ \times 30 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 4.0 M urea (pH 8.0), and developed using the same buffer solution as was used for equilibration, to obtain 10 ml of eluate as hGH fractions possessing diketone of the methionine residue. This eluate was added into 10 ml of 2 M formic acid, 10 M sodium formate, 6 M urea solution, and 80 mM 3,4-diaminobenzoic acid solution while stirring well, then allowed to react for 3 days at 30°C while stirring slowly. Following the reaction, it was then applied at a flow rate of 500 ml/h to a Sephadex G-25 column (4.6 cm ϕ \times 60 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 4.0 M urea (pH 8.0), to collect 100 ml of hGH fractions. Subsequently, in high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.) this solution was made to flow through and adsorb to a DEAE-5PW column (2.15 cm \times 15 cm, Tosoh Corporation), followed by elution at a flow rate of 7.5 ml/minute for 70 minutes in a pH gradient of 70 to 85% B generated with A = 50 mM Tris/HCl + 2.5 M urea (pH 8.0) and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid] + 2.5 M urea (pH 4.0), to obtain 6.0 mg of hGH fractions.

Example 10 (Activation of 20K-hGH possessing methionine residue (Met-20K-hGH))

40 g of cells obtained via the method described in Reference 2 of Unexamined Kokai Application Heisei 10-234386 was suspended in 100 milliliters of PBS (phosphate buffered saline), then the cells were

disrupted with 5 minutes of sonication on a sonifier (Branson Ultrasonics Corporation). The lysate was subjected to centrifugation (10,000 rpm for 20 minutes) and the supernatant was discarded, to obtain the pellet.

5 Following the addition of 2 liters of 50 mM Tris/HCl and 8 M guanidine hydrochloride solution (pH 8.0) to the pellet to dissolve it, centrifugation (10,000 rpm for 120 minutes) was performed. 24 liters of 50 mM Tris/HCl, 0.28 mM GSSG, 1.4 mM GSH, and 0.7 M arginine

10 (pH 8.0) was added to 2 liters of supernatant obtained, whereupon activation was conducted for 1 day at 4°C.

Example 11 (Purification of Met-20K-hGH)

The solution which underwent activation obtained

15 in Example 10 was desalted and concentrated in a Minitan ultrafiltration system (PTGC membrane, Millipore Corporation) while adding 20 mM Tris/HCl and 2.5 M urea (pH 8.0) until the electric conductivity was no greater than 10 mS/cm, whereupon the concentrate

20 obtained was subjected to centrifugation (10,000 rpm for 20 minutes), to yield 150 milliliters of supernatant. Subsequently, this supernatant was applied to a HiLoad™ Q Sepharose 16/10 HP column (1.6 cmφ × 10 cm, Pharmacia Biotech, Inc.) equilibrated with

25 50 mM Tris/HCl and 2.5 M urea/10% acetonitrile (pH 8.2) for adsorption, then underwent a thorough wash, followed by elution at a flow rate of 3.0 milliliters/minute with a sodium chloride gradient from 0 to 0.18 M, to obtain 28 milliliters of eluate as Met-

30 20K-hGH fractions. These fractions were further concentrated and desalted using a Centriplus-10 (Millipore Corporation) to yield 15 milliliters of concentrate. This liquid was readsorbed onto a HiLoad™ Q Sepharose 16/10 HP column (1.6 cmφ × 10 cm, Pharmacia

35 Biotech, Inc.) equilibrated with 50 mM Tris/HCl and 2.5

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M urea/10% acetonitrile (pH 8.2), and underwent a thorough washing, followed by elution at a flow rate of 3.0 milliliters/minute for 60 minutes in a pH gradient of 0 to 100% B generated with A = 50 mM Tris/HCl, 2.5 M urea, and 10% acetonitrile (pH 8.2) and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid], 2.5 M urea, and 10% acetonitrile (pH 4.0), to obtain 12 milliliters of Met-20K-hGH fractions. To this eluate, 0.6 milliliters of 2 M Tris/HCl (pH 7.8) was added to adjust the pH to 7.2, followed by concentration using a Centriplus-10 (Millipore Corporation). 0.5 milliliters of this concentrate was added to a Superdex™ 75 HR 10/30 (1.0 cmφ × 30 cm, Pharmacia Biotech, Inc.) equilibrated with PBS containing 10% ethanol, to obtain 7.5 milliliters of Met-20K-hGH fractions.

Example 12 (Removal of N-terminal Met)

6 milliliters of the Met-20K-hGH solution obtained in Example 11 was applied to a Sephadex G-25 column (10 mm ID × 30 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 8 M urea (pH 8.0) and the eluted Met-20K-hGH fractions were collected, followed by further concentration to 2 milliliters using an ultrafiltration system (Diaflow Membrane YM10, 25 mm, Amicon, Inc.). 0.5 milliliters of 2.5 M glyoxylic acid, 40 mM cupric sulfate, and 50% pyridine solution was added to this solution, stirred well, and allowed to react for 60 minutes at 25°C. Subsequently, this reaction solution was applied to a Sephadex G-25 column (10 mm ID × 40 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 4 M urea (pH 8.0) to collect 4 milliliters of eluate as 20K-hGH fractions possessing diketone of methionine residue. 20 milliliters of 1.2 M acetic acid, 2.4 M sodium formate, 3.6 M urea, and 48 M 3,4-diaminobenzoic acid was added

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to this solution, stirred well, and was allowed to react for 65 hours at 30°C. Following the reaction, the reaction product was applied to a Sephadex G-25 column (20 mm ID × 40 cm, Pharmacia Biotech, Inc.) equilibrated with 20 mM Tris/HCl and 4 M urea (pH 8.0), to collect 20K-hGH fractions, whereupon high speed liquid chromatography (Gilson HPLC system, Gilson Company, Inc.) was conducted, wherein the fractions were applied to a HiLoad™ Q Sepharose 16/10 HP column (1.6 cmφ × 10 cm, Pharmacia Biotech, Inc.) equilibrated with 50 mM Tris/HCl and 2.5 M urea/10% acetonitrile (pH 8.2) for adsorption, and made to undergo a thorough wash, followed by elution at a flow rate of 3.0 milliliters/minute for 60 minutes in a pH gradient of 0 to 100% generated with A = 50 mM Tris/HCl, 2.5 M urea, and 10% acetonitrile (pH 8.2), and B = 50 mM MES [2-(N-morpholino)-ethane sulfonic acid], 2.5 M urea, and 10% acetonitrile (pH 4.0), to obtain 12 milliliters of 20K-hGH fractions. 0.6 milliliters of 2 M Tris/HCl (pH 7.8) was added to this eluate to adjust the pH to 7.2, whereupon it was concentrated using a Centriplus-10 (Millipore Corporation). 0.5 milliliters of this concentrate was added to a Superdex™ 75 HR 10/30 (1.0 cmφ × 30 cm, Pharmacia Biotech, Inc.) equilibrated with PBS containing 10% ethanol, followed by elution with PBS containing 10% ethanol to obtain 7.5 milliliters of 20K-hGH fractions.

Example 13 (Characterization of 20K-hGH)

(a) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Perkin Elmer Applied Biosystems Model 477A). The 20K-hGH N-terminal amino acid sequence thus obtained matched the 20K-hGH N-terminal amino acid sequence that was deduced from

the cDNA base sequence (Table 4).

Table 4

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid deduced from 20K-hGH base sequence
1	Phe (642)	Phe
2	Pro (504)	Pro
3	Thr (342)	Thr
4	Ile (410)	Ile
5	Pro (200)	Pro
6	Leu (378)	Leu
7	Ser (95)	Ser
8	Arg (170)	Arg
9	Leu (285)	Leu
10	Phe (262)	Phe

- 5 1) Phenylthiohydantoin
Analysis performed using 1 nmol.

(b) Amino acid composition analysis

- 10 The amino acid composition was determined using an amino acid analyzer (System 6300, Beckman, Inc.). The amino acid composition of the 20K-hGH obtained in Example 12 matched the amino acid composition deduced from the cDNA base sequence of 20K-hGH (Table 5).

15 Table 5

Amino acid	Number of residues per mole	Value deduced from 20K-hGH base sequence
Asx	20.2	20
Thr ¹⁾	9.7	10
Ser ¹⁾	16.5	17
Glx	22.0	22
Pro	6.9	7

Gly	8.0	8
Ala	6.2	6
Cys ²⁾	N.D.	4
Val	7.0	7
Met	2.9	3
Ile	6.5	7
Leu	24.3	25
Tyr	5.9	6
Phe	12.2	12
His	3.1	3
Lys	7.1	7
Arg	10.7	11
Trp	N.D.	1

Acid hydrolysis (mean value of 24-hours and 48-hour hydrolysis, at 110°C, with 6N HCl-1% phenol). Analysis was performed using approximately 20 µg.

1) Value extrapolated at 0 hours.

5 2) Not detected.

Example 14 (Measurement of 20K-hGH activity)

The growth promoting activity of the 20K-hGH obtained in Example 12 on Nb2 cells [Journal of
10 Clinical Endocrinology and Metabolism, Vol. 51, p. 1058 (1980)] was confirmed.

Example 15 (Manufacture of human BTC which possesses methionine residue (human Met-BTC))

15 In accordance with Examples 4 through 6, 8, and 13 of Unexamined Kokai Application Heisei 6-87894 (EP-A-0555785), human Met-BTC was manufactured in the following method.

20 (Construction of human BTC cDNA expression plasmid in *E. coli*)

The 0.6 Kb EcoRI-BamHI fragment, which codes for

human pro-BTC (1-147 amino acid residue), was isolated from Plasmid pTB1515 described in Example 5 of Unexamined Kokai Application Heisei 6-87894 (EP-A-0555785). Upon ligating a synthetic adapter with ATG translation initiation codon (5'-TATGGATGGG-3'; 5'-AATTCCCATCCA-3') into the EcoRI site of the 0.6 Kb fragment, the 0.6 Kb NdeI-BamHI fragment generated was inserted into plasmid pET-3c containing a T7 promoter (Gene, Vol. 56, p. 125 (1987)), to construct plasmid pTB1505.

In order to obtain DNA fragments that code for the 80 amino acid residues in human BTC (1 (Asp) through 80 (Tyr) in Figures 10-1 and 10-2 (Unexamined Kokai Application Heisei 6-87894 (EP-A-0555785))), PCR (polymerase chain reaction) was performed using plasmid pTB1505 as the template and two oligonucleotides (5'-ATACATATGGATGGGAATTCCA-3'; 5'-CCGGATCCTAGTAAAACAAGTCAACTCT-3') as the primer. The product was digested with NdeI and BamHI, followed by fractionation via electrophoresis with 2.0% agarose gel, to isolate the targetted 0.25 Kb DNA fragment. This 0.25 Kb NdeI-BamHI fragment was ligated to the downstream of the T7 promoter of pET-3c using T4DNA ligase, to obtain plasmid pTB1516 (See Figure 13 of Unexamined Kokai Application Heisei 6-87894 (EP-A-0555785))).

(Expression of human Met-BTC in *E. coli*)

E. coli MM294 was lysogenized by a recombinant lambda-phage containing the T7 RNA polymerase gene (Studier, supra). Subsequently, plasmid pLysS was introduced into this *E. coli* MM294(DE3), to obtain *E. coli* MM294(DE3)/pLysS. The plasmid pTB1516 obtained in the aforementioned Reference was introduced into these cells, to obtain *E. coli* MM294(DE3)/pLysS, pTB1516.

This transformant was cultured with shaking for 8

hours at 37°C, in a 2 liter flask containing 1 liter of LB culture medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride) containing 50 µg/ml of ampicillin and 15 µg/ml of chloramphenicol. The culture solution thus
5 obtained was transferred to a 50 liter fermenter containing 19 liters of fermentation medium (1.68% dibasic sodium phosphate, 0.3% monobasic potassium phosphate, 0.1% ammonium chloride, 0.05% sodium chloride, 0.05% magnesium sulfate, 0.02% defoaming
10 agent, 0.00025% ferrous sulfate, 0.0005% thiamine hydrochloride, 1.5% glucose, 1.5% casamino acid), to start aeration/agitation at 30°C. When the turbidity of the culture solution reached approximately 500 Klett units, 100 mg/liter of isopropyl-β-D-
15 thiogalactopyranoside (IPTG) was added, and cultivation was continued, to be terminated 7 hours later. This culture solution was subjected to centrifugation to obtain approximately 340 g of wet cells, which were then frozen for storage at -80°C.

20 This transformed *E. coli* (MM294(DE3)/pLySS, pTB1516) was deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry (NIBH) on April 21,
25 1992, under accession number FERM BP-3836, and with the Institution for Fermentation, Osaka (IFO) on April 16, 1992, under accession number IFO 15282.

Upon dissolving 10 mg of the human β-cellulin possessing N-terminal methionine residue (Met-BTC),
30 obtained via the aforementioned method, in 4 ml of 3M urea solution, a mixture of 0.25 ml 80 mM cupric sulfate, 0.25 g glyoxylic acid, and 0.5 ml pyridine was added, to be allowed to react for 1 hour at 25°C. Upon completion of the reaction, the reaction solution was
35 applied to a Sephadex G-25 column (25 mm ID × 600 mmL)

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equilibrated with 2.5 M urea + 50 mM phosphate buffer solution (pH 6.0), developed in the solution used for equilibration at a flow rate of 6 ml/minute, and the BTC fractions possessing diketone of the methionine residue were pooled. Subsequently, an equal volume of 2 M acetic acid, 4 M sodium formate, and 3 M urea solution was added to these fractions, followed by the addition of 3,4-diaminobenzoic acid so that the concentration was 40 mM, whereupon deaeration was conducted and a nitrogen gas seal applied, to allowed reaction to proceed for 5 days at 25°C. Upon completion of the reaction, the reaction solution was applied to a Sephadex G-25 column (46 mm ID × 600 mmL) equilibrated with 50 mM phosphate buffer solution (pH 6.0), developed in the buffer solution used for equilibration at a flow rate of 10 ml/minute, and the BTC fractions which do not possess N-terminal methionine pooled. The pooled fractions, upon being adjusted to pH 6.0, were adsorbed to a CM-5PW (21.5 mm ID × 150 mmL, Tosoh Corporation) equilibrated with 50 mM phosphate buffer solution + 0.1 M NaCl + 2.5 M urea (pH 5.0) to be eluted at a flow rate of 6 ml/minute for 60 minutes at a step gradient of 0 to 100% B (B = 50 mM boric acid buffer solution + 0.1 M NaCl + 2.5 M urea, pH 9.0), and the BTC fractions pooled. The BTC fractions were further adsorbed onto a C4P-50 (10 mm ID × 250 mmL, Showa Denko K.K.) equilibrated with 0.1% TFA, then eluted at a flow rate of 2 ml/minute for 40 minutes on a step gradient of 20 to 60% B (B = 80% acetonitrile/0.1% TFA). Following pooling, the BTC fractions were lyophilized, to yield approximately 2.6 mg of BTC.

Example 16 (Characterization of BTC)

a) Analysis using SDS polyacrylamide gel

electrophoresis

The BTC obtained in Example 15 was suspended in sample buffer [Laemmli, Nature, 227, 680 (1970)] and heated for 1 minute at 100°C, followed by electrophoresis using Multigel 15/25 (Daichi Pure Chemicals Co., Ltd.). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, whereupon a single band of protein was observed, thereby confirming that the purified product was virtually monomeric (Figure 2).

b) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Perkin Elmer Applied Biosystems Model 477A). The amino acid sequence of BTC thus obtained matched the BTC N-terminal amino acid sequence that was deduced from the BTC cDNA base sequence (Table 6).

Table 6

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid (pmol) deduced from BTC base sequence
1	Asp (261)	Asp
2	Gly (457)	Gly
3	Asn (300)	Asn
4	Ser (107)	Ser
5	Thr (75)	Thr
6	Arg (181)	Arg
7	Ser (121)	Ser
8	Pro (245)	Pro
9	Glu (55)	Glu
10	Thr (71)	Thr
11	Asn (133)	Asn
12	Gly (149)	Gly

13	Leu (132)	Leu
14	Leu (155)	Leu
15	N.D.	Cys
16	Gly (111)	Gly
17	Asp (70)	Asp
18	Pro (65)	Pro
19	Glu (29)	Glu
20	Glu (64)	Glu

Analysis was performed using 1 nmol.

N.D.: Not detected

1) Phenylthiohydantoin

5 c) Amino acid composition analysis

The amino acid composition was determined using an amino acid analyzer (Beckman System 6300E). The amino acid composition obtained matched the amino acid composition deduced from the BTC cDNA base sequence

10 (Table 7).

Table 7

Amino acid	Number of residues per mole	Value deduced from BTC base sequence
Asx	7.0	7
Thr ¹⁾	6.1	6
Ser ¹⁾	4.8	5
Glx	9.3	9
Pro	3.8	4
Gly	7.1	7
Ala	4.0	4
Cys ²⁾	N.D.	8
Val	3.9	4
Met	0	0
Ile	1.9	2
Leu	3.0	3
Tyr	3.7	4

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Phe	3.3	3
His	2.3	2
Lys	5.0	5
Arg	6.9	7
Trp	0	0

Acid hydrolysis (mean value of 24-hours and 48-hour hydrolysis, at 110°C, with 6N hydrochloric acid and 1% phenol).

1) Value extrapolated at 0 hours.

5 2) Not detected

Analysis was performed using ca 20 µg.

d) C-terminal amino acid analysis

10 The C-terminal amino acid was determined using an amino acid analyzer (Beckman System 6300E). The BTC thus obtained matched the C-terminal amino acid deduced from the cDNA base sequence (Table 8).

Table 8: C-terminal amino acid analysis

15

BTC	C-terminal amino acid	Recovery rate (%)
	Tyr	44.6

Phase hydrazinolysis (at 100°C for 3.5 hours)

Analysis was performed using 15 nmol.

e) Biological activity of BTC

20 The purified product was measured for activity according to the method described in Molecular Cell Biology, 8, 588 (1988) using BALB/C3T3 A31-714 Clone 4 (International Journal of Cancer, 12, 463 (1973), whereupon the activity of said product was confirmed to
25 be equivalent to that of the reference material.

Example 17

Upon dissolving 50 mg of human interleukin-2

possessing N-terminal methionine residue (Met-IL-2),
obtained according to the method in Reference 5 of
Unexamined Kokai Application Heisei 10-72489 (EP-A-
812856), into 40 ml of 4 M urea solution, a mixture of
5 2.5 ml 100 mM cupric sulfate, 2.5 g glyoxylic acid, and
5.0 ml pyridine was added, to be allowed to react for 1
hour at 25°C. After the reaction, the reaction
solution was applied to a Sephadex G-25 column (46 mm
ID × 600 mmL) equilibrated with 10 mM phosphate buffer
10 solution + 2.5 M urea (pH 5.0), developed in the buffer
used for equilibration at a flow rate of 10 ml/minute,
and IL-2 fractions possessing diketone of the
methionine residue were pooled. Subsequently, an equal
volume of 2 M acetic acid, 4 M sodium formate, and 3 M
15 urea solution was added to these fractions, followed by
the addition of 3,4-diaminobenzoic acid to make the
concentration 40 mM, whereupon deaeration was conducted
and a nitric gas seal was applied, and the reaction
allowed to proceed for 5 days at 25°C. Upon completion
20 of the reaction, the reaction solution was applied to a
Sephadex G-25 column (46 mm ID × 600 mmL) equilibrated
with 10 mM phosphate buffer solution + 2.5 M urea (pH
5.0), developed in the buffer solution used for
equilibration at a flow rate of 10 ml/minute, and IL-2
25 fractions which do not possess N-terminal methionine
pooled. The pooled IL-2 fractions were adsorbed onto
SP-5PW (21.5 mm ID × 150 mmL, Tosoh Corporation)
equilibrated with 25 mM phosphate buffer solution (pH
7.0), followed by elution at a flow rate of 6 ml/minute
30 for 60 minutes on a step gradient of 30 to 80% B (B =
25 mM phosphate buffer solution, pH 8.0), to yield 17.3
mg of IL-2 fractions.

Example 18 (Characterization of IL-2)

35 a) Analysis using SDS polyacrylamide gel

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electrophoresis

The IL-2 obtained in Example 17 was suspended in the sample buffer [Laemmli, Nature, 227, 680 (1970)] and heated for 1 minute at 100°C, followed by electrophoresis using Multigel 15/25 (Daiichi Pure Chemicals Co., Ltd.). Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, whereupon a single band of protein was observed, thereby confirming that the purified product was virtually monomeric (Figure 3).

b) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Perkin Elmer Applied Biosystems Model 477A). The amino acid sequence of IL-2 thus obtained matched the IL-2 N-terminal amino acid sequence that was deduced from the IL-2 cDNA base sequence (Table 9).

Table 9

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid deduced from IL-2 base sequence
1	Ala (701)	Ala
2	Pro (354)	Pro
3	Thr (359)	Thr
4	Ser (122)	Ser
5	Ser (128)	Ser
6	Ser (78)	Ser
7	Thr (46)	Thr
8	Lys (176)	Lys
9	Lys (61)	Lys
10	Thr (40)	Thr

Analysis was performed using 1 nmol.

1) Phenylthiohydantoin

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c) Amino acid composition analysis

The amino acid composition was determined using an amino acid analyzer (Beckman System 6300E). The amino acid composition obtained matched the amino acid composition deduced from the IL-2 cDNA base sequence (Table 10).

Table 10

10

Amino acid	Number of residues per mole	Value deduced from IL-2 base sequence
Asx	11.8	12
Thr ¹⁾	12.9	13
Ser ¹⁾	7.0	8
Glx	18.4	18
Pro	4.8	5
Gly	2.0	2
Ala	4.8	5
Cys ²⁾	N.D.	3
Val	3.5	4
Met	3.8	4
Ile	7.7	9
Leu	22.0	22
Tyr	2.8	3
Phe	5.6	6
His	2.9	3
Lys	10.3	11
Arg	3.7	4
Trp	0.9	1

Acid hydrolysis (mean value of 24-hours and 48-hour hydrolysis, at 110°C, with 6N hydrochloric acid - 4% thioglycolic acid).

1) Value extrapolated at 0 hours.

15 2) Not detected

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d) C-terminal amino acid analysis

The C-terminal amino acid was determined using an amino acid analyzer (Beckman System 6300E). The IL-2 thus obtained matched the C-terminal amino acid deduced from the cDNA base sequence (Table 11).

Table 11: C-terminal amino acid analysis

IL-2	C-terminal amino acid	Recovery rate (%)
	Thr	32.5

Phase hydrazinolysis (at 100°C for 3.5 hours)

Analysis was performed using 15 nmol.

e) Biological activity of IL-2

Measurement of biological activity was performed according to the method using IL-2 dependent cells described by Hinuma et al. [Biochem. Biophys. Res. Commun., 109, 363 (1982)], whereupon the biological activity was confirmed to be equivalent to that of the reference material.

Example 19 (Activation of Met-hGH)

4 liters of 50 mM Tris/acetic acid, 8 M guanidine hydrochloride solution (pH 8.5) was added to 1 kg of the cells obtained in Reference 2 to dissolve the cells, followed by centrifugation (10,000 rpm). 44 liters of 50 mM Tris/acetic acid, 1.09 mM reduced glutathione, 0.055 mM oxidized glutathione, 109 mM arginine, and 4.36 M urea solution (pH 8.0) was added to approximately 4 liters of supernatant obtained, and the activation was allowed to proceed for 3 days at 4°C. Following the activation, the solution was concentrated and desalted in a Pellicon cassette system (Biomax 8 membrane, Millipore Corporation) while adding approximately 25 liters of 20 mM Tris/acetic acid and

2.5 M urea solution (pH 8.0), until the electrical conductivity was no greater than 5 mS/cm. Subsequently, desalting was conducted again while adding approximately 35 liters of 20 mM Tris/acetic acid solution (pH 8.0), followed by centrifugation (10,000 rpm) to obtain supernatant. Subsequently, the supernatant was adsorbed onto a DEAE-Toyopearl 650 M column (30 cmφ × 60 cm, Tosoh Corporation) equilibrated with 20 mM Tris/acetic acid solution (pH 8.0), then thoroughly washed in 20 mM Tris/acetic acid solution (pH 8.0) and 20 mM Tris/acetic acid, 25 mM sodium chloride solution (pH 8.0), followed by elution with 20 mM Tris/acetic acid and 55 mM sodium chloride solution (pH 8.0), to obtain 50 liters of eluate as Met-hGH fractions. This eluate was concentrated and desalted in a Pellicon cassette system (Biomax 8 membrane, Millipore Corporation) to obtain Met-hGH.

Example 20 (Activation of Met-hGH)

After adding 4 liters of 50 mM Tris/HCl and 8 M guanidine hydrochloride solution (pH 8.5) to 1 kg of cells obtained in Reference 2, the cells were dissolved, whereupon centrifugation (10,000 rpm) was conducted. To approximately 4 liters of supernatant obtained, 44 liters of 50 mM Tris/acetic acid, 5.45 mM cysteine hydrochloride monohydrate, 109 mM arginine, and 4.91 M urea solution (pH 8.0) was added, and the activation was conducted for 3 days at 4°C. The amount of activated Met-hGH obtained was approximately 1.2 times more than that obtained in Example 19. Following the activation, the solution was concentrated and desalted in a Pellicon cassette system (Biomax 8 membrane, Millipore Corporation) while adding 25 liters of 20 mM Tris/acetic acid and 2.5 M urea solution (pH 8.0) until the electric conductivity was no greater than 5 mS/cm.

Desalting was conducted again while adding 35 liters of 20 mM Tris/acetic acid solution (pH 8.0), whereupon centrifugation (10,000 rpm) was performed to obtain supernatant. Subsequently, the supernatant was applied to a DEAE-Toyopearl 650 column (30 cm ϕ \times 60 cm, Tosoh Corporation) equilibrated with 20 mM Tris/acetic acid solution (pH 8.0) for adsorption, followed by a thorough wash using 20 mM Tris/acetic acid solution (pH 8.0) and 20 mM Tris/acetic acid, and 25 mM sodium chloride solution (pH 8.0), followed by elution with 20 mM Tris/acetic acid and 55 mM sodium chloride solution (pH 8.0), to obtain 50 liters of eluate as Met-hGH fractions. This eluate was concentrated and desalted in a Pellicon cassette system (Biomax 8 membrane, Millipore Corporation) to obtain Met-hGH.

Example 21 (Activation of Met-hGH)

After adding 5 milliliters of 50 mM Tris/acetic acid and 8 M guanidine hydrochloride solution (pH 8.5) to 1.25 g of cells obtained in Reference 2, the cells were dissolved, whereupon centrifugation (10,000 rpm) was conducted. To approximately 5 milliliters of supernatant obtained, 55 milliliters of 50 mM Tris/acetic acid, 5.45 mM N-acetyl-L-cysteine, 109 mM arginine, and 4.91 M urea solution (pH 8.0) was added, and activation was conducted for 3 days at 4°C. The resulting activation efficiency observed was equivalent to that observed in Example 20, wherein cysteine hydrochloride monohydrate was added.

Example 22 (Activation of Met-hGH)

5 milliliters of 50 mM Tris/acetic acid and 8 M guanidine hydrochloride solution (pH 8.5) was added to 1.25 g of cells obtained in Reference 2 to dissolve the cells, and centrifugation (10,000 rpm) was conducted.

To approximately 5 milliliters of supernatant obtained, 55 milliliters of 50 mM Tris/acetic acid, 5.45 mM cysteamine hydrochloride, 109 mM arginine, and 4.91 M urea solution (pH 8.0) was added, and activation was
5 conducted for 3 days at 4°C. The resulting activation efficiency observed was equivalent to that observed in Example 20, where cysteine hydrochloride monohydrate was added.

10 Example 23

Human neurotrophin-3 possessing N-terminal methionine residue (Met-NT-3) was manufactured according to the method described in Reference 3 of Unexamined Kokai Application Heisei 10-72489 (EP-A-
15 812856).

50 mg of human neurotrophin-3 possessing methionine on the N-terminal (Met-NT-3) was dissolved into 8 ml of 3 M urea solution, whereupon a mixture comprising 0.4 ml of 0.2 M cupric sulfate, 0.5 g of
20 glyoxylic acid, and 1 ml of pyridine was added to bring the total to 10 ml, whereupon it was allowed to react for 1 hour at 25°C. After termination of the reaction, the reaction solution was made to flow through a Sephadex G-25 column (25 mm ID × 66 mmL) equilibrated
25 with 2.5 M urea + 10 mM phosphate buffer solution (pH 6.0), the solution used for equilibration developed at a flow rate of 4 ml/minute, and NT-3 fractions possessing diketone of the methionine residue were pooled. Subsequently, an equal volume of 2 M acetic
30 acid, 4 M sodium formate, and 3 M urea solution was added to these fractions, followed by the addition of 3,4-diaminobenzoic acid to bring the concentration to 40 mM, to be allowed to react for 5 days at 25°C. After the reaction, the reaction solution was made to
35 flow through a Sephadex G-25 column (46 mm ID × 600

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mmL) equilibrated with 2.5 M urea + 10 mM phosphate buffer solution (pH 6.0), the buffer solution used for equilibration developed at a flow rate of 10 ml/minute, and human neurotrophin-3 without N-terminal methionine residue (NT-3) fractions were pooled. After adjusting the pH of the pooled NT-3 fractions to 5.0, said fractions were applied to a CM-5PW (21.5 mm ID × 150 mmL, Tosoh Corporation) equilibrated with 50 mM phosphate buffer solution + 0.2 M NaCl + 2.5 M urea (pH 5.0) for adsorption, followed by elution at a flow rate of 6 ml/minute on a step gradient of 0 to 100% B (B = 50 mM phosphate buffer solution + 0.2 M NaCl + 2.5 M urea, pH 8.0) for 60 minutes, to pool NT-3 fractions. Further, the NT-3 fractions were made to adsorb to a C4P-50 (21.5 mm ID × 300 mmL, Showa Denko K.K.) equilibrated with 0.1% TFA, followed by elution at a flow rate of 5 ml/minute on a step gradient of 20 to 60% B (B = 80% acetonitrile/0.1% TFA) for 40 minutes. After pooling, the NT-3 fractions were lyophilized, to yield approximately 5 mg of NT-3 in lyophilized powder form.

Example 24 (Characterization of NT-3)

a) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Applied Biosystems Model 477A). The amino acid sequence thus obtained matched the NT-3 N-terminal amino acid sequence that was deduced from the cDNA base sequence (Table 12).

Table 12

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid deduced from NT-3 base sequence
1	Tyr (410)	Tyr

2	Ala (521)	Ala
3	Glu (155)	Glu
4	His (213)	His
5	Lys (587)	Lys
6	Ser (91)	Ser
7	His (161)	His
8	Arg (318)	Arg
9	Gly (214)	Gly
10	Glu (108)	Glu
11	Tyr (104)	Tyr
12	Ser (50)	Ser
13	Val (208)	Val
14	N.D.	Cys
15	Asp (99)	Asp
16	Ser (41)	Ser
17	Glu (24)	Glu
18	Ser (27)	Ser
19	Leu (63)	Leu
20	Trp (26)	Trp

Analysis was conducted using 1 nmol.

N.D.: Not detected.

*) Phenylthiohydantoin

5 b) Amino acid composition analysis

The amino acid composition was determined using an amino acid analyzer (Beckman System 6300E). The amino acid composition thus obtained matched the amino acid composition deduced from the NT-3 cDNA base sequence

10 (Table 13).

Table 13

Amino acid	Number of residues per mole	Value deduced from NT-3 base sequence
Asx	11.0	11
Thr*)	8.7	9

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Ser ^{*)}	10.4	12
Glx	11.0	11
Pro	1.7	2
Gly	8.0	8
Ala	4.8	5
Cys	N.D.	6
Val	8.7	9
Met	0	0
Ile	6.7	7
Leu	5.0	5
Tyr	5.1	5
Phe	1.2	1
His	4.3	4
Lys	9.7	10
Arg	9.6	10
Trp	3.8	4

Acid hydrolysis (mean value of 24-hour and 48-hour hydrolysis, at 110°C, with 6N hydrochloric acid - 4% thioglycolic acid).

N.D.: Not detected

5 *) Value extrapolated at 0 hours.

Analysis was performed using ca 20 µg.

d) C-terminal amino acid analysis

10 The C-terminal amino acid was determined using an amino acid analyzer (Beckman System 6300E). The amino acid thus obtained matched the C-terminal amino acid deduced from the cDNA base sequence (Table 14).

Table 14

15

NT-3	C-terminal amino acid	Recovery rate (%)
	Thr	42.0

Phase hydrazinolysis (at 100°C for 3.5 hours)

Analysis was performed using 15 nmol.

e) Biological activity of NT-3

The biological activity of the NT-3 obtained in Example 23 was measured using DRG (dorsal root ganglia, taken from the embryos of fertilized chicken eggs upon embryogenesis following 8 to 10 days of incubation at 37.5°C) bioassay, whereupon it was confirmed that said NT-3 possessed a degree of activity equivalent to that of NT-3 obtained from CHO cells.

Example 25

After adding 6 M urea solution to 14.75 ml of the Met-hGH solution obtained in Example 2 to bring the total to 60 ml, a mixture comprising 1.2 ml of 0.5 M cupric sulfate, 3.75 g of glyoxylic acid, and 7.5 ml of pyridine was added to bring the total to 75 ml, whereupon it was allowed to react for 1 hour at 25°C. After termination of the reaction, the reaction solution was made to flow through a Sephadex G-25 column (4.6 cm ID × 60 cmL) equilibrated with 4 M urea + 20 mM Tris buffer solution (pH 8.0), the buffer solution used for equilibration developed at a flow rate of 10 ml/minute, to pool hGH fractions possessing diketone of the methionine residue. Subsequently, an equal volume of 2 M acetic acid, 4 M sodium formate, and 4 M urea solution was added to these fractions, followed by the addition of 3,4-diaminobenzoic acid to bring the concentration to 40 mM, to be allowed to react for 4 days at 30°C. After termination of the reaction, the reaction solution was made to flow through a Sephadex G-25 column (11.3 cm ID × 80 cmL) equilibrated with 4 M urea + 20 mM Tris buffer solution (pH 8.0), the buffer solution used for equilibration developed at a flow rate of 30 ml/minute, and hGH fractions without N-terminal methionine residue were

pooled. The pooled hGH fractions were applied to a DEAE-5PW (5.5 cm ID × 20 cmL, Tosoh Corporation) equilibrated with 50 mM Tris buffer solution + 2.5 M urea (pH 8.0) for adsorption, followed by elution at a flow rate of 15 ml/minute on a step gradient of 0 to 100% B (B = 50 mM MES + 2.5 M urea, pH 4.0) for 60 minutes, to obtain approximately 60 mg of hGH.

Example 26 (Preparation of human apelin-36 structural gene)

The 6 types of DNA fragments shown in Figure 4 (#1 and #5: Greiner Japan Co. Ltd.; #2 and #6: Kikotec Co.; #3 and #4: Amersham Pharmacia Biotech) were used to prepare the apelin-36 structural gene (Figure 5).

a) Phosphorylation of DNA oligomer

With the exclusion of oligomers #1 and #6, 1 µg each of the remaining 4 types of oligomers destined to become 5' terminals was allowed to react for 1 hour at 37°C in 100 µL of phosphorylation reaction solution [50 mM Tris/HCl (pH 7.6), 10 mM MgCl₂, 1 mM spermidine, 10 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 1 mM ATP, 10 units of T4 polynucleotide kinase (Nippon Gene)], to phosphorylate the 5' terminals. After phenol extraction, the aqueous phase was recovered, 2 volumes of ethanol was added, followed by cooling to -70°C, then by centrifuge to precipitate the DNA.

b) Ligation of DNA fragments

The phosphorylated DNA fragments obtained in a) above were combined with 1 µg each of #1 and #2 to make a total of 120 µL. After maintaining this mixture at 80°C for 10 minutes, said mixture was cooled slowly to room temperature for annealing the fragments. TaKaRa DNA Ligation Kit ver. 2 (Takara Shuzo) was used to

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perform the ligation reaction. After adding 30 μ L of Solution II to 30 μ L of the annealing solution and mixing well, 60 μ L of Solution I was added, and allowed to react for 1 hour at 37°C to perform the ligation.

- 5 After phenol extraction, the aqueous phase was recovered, 2 volumes of ethanol was added, followed by cooling to -70°C, then by centrifuge to precipitate the DNA.

10 c) Phosphorylation of the 5' terminal

- The precipitate was dissolved in 10 μ L of TE buffer solution (10 mM Tris/HCl (pH 8.0) and 1 mM EDTA), then allowed to react for 1 hour at 37°C in 100 μ L of phosphorylation reaction solution [50 mM Tris/HCl (pH 7.6), 10 mM MgCl₂, 1 mM spermidine, 10 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 1 mM ATP, 10 units of T4 polynucleotide kinase (Nippon Gene)], to phosphorylate the 5' terminals. After phenol extraction, the aqueous phase was recovered, 2 volumes of ethanol was added, followed by cooling to -70°C, then by centrifuge to precipitate the DNA, which was dissolved into 20 μ L of TE buffer solution.

25 Example 27 (Preparation of human apelin-36 expression plasmid)

- PTB960-2 (EP-A-499990: Koyama et al, Journal of Biotechnology, 32, p. 273) was digested with XbaI and AvaI, followed by 1% agarose electrophoresis, to extract approximately 4.4 Kbp of DNA fragments using QIAquick Gel Extraction Kit (QIAGEN), which were dissolved into 25 μ L of TE buffer solution. These XbaI and AvaI fragments of pTB960-2 and the human apelin-36 structural gene prepared using the aforementioned method were subjected to ligation reaction using TaKaRa DNA Ligation Kit ver. 2 (Takara Shuzo). Namely, 1 μ L

of the pTB960-2 XbaI and AvaI fragment solution was mixed with 4 μ L of human apelin-36 structural gene solution, followed by the addition of 5 μ L of Solution I, to be allowed to react for 30 minutes at 16°C to perform the ligation. 10 μ L of the ligation solution was used to transform *E. coli* JM109 competent cells (Toyobo Co. Ltd.), which were then plated on LB agar medium containing 10 μ g/mL tetracycline and cultured for 1 day at 37°C, whereupon the tetracycline-resistant colonies generated were selected. These transformants were cultured on LB medium overnight, whereupon QIAprep 8 Miniprep Kit (QIAGEN) was used to prepare plasmid pTB960-13. The base sequence for this human apelin-36 structural gene fragment was verified using an Applied Biosystems Model 377 DNA Sequencer. Plasmid pTB960-13 was used to transform *E. coli* BL21(DE3) strain (Novagen, Inc.), which was then plated on LB agar medium containing 10 μ g/mL tetracycline, and cultured for 1 day at 37°C, to obtain human apelin-36-CS23 fusion protein expression strain BL21(DE3)/pTB960-13 (Figure 6). This transformant *E. coli* BL21(DE3)/pTB960-13 was deposited with the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology of the Ministry of International Trade and Industry on December 2, 1998, under accession number FERM BP-6590, and with the Institution for Fermentation, Osaka (IFO) on November 11, 1998, under accession number IFO 16220.

Example 28

The transformed cells obtained in Example 27 were cultured with shaking for 8 hours at 37°C within a 2-liter flask containing 1 L of LB medium (1% peptone, 0.5% yeast extract, 0.5% sodium chloride) containing 5.0 mg/L tetracycline. The culture obtained was

transferred to a 50 L fermenter containing 19 liters of fermentation medium (1.68% dibasic sodium phosphate, 0.3% monobasic potassium phosphate, 0.1% ammonium chloride, 0.05% sodium chloride, 0.05% magnesium sulfate, 0.02% antifoaming agent, 0.00025% ferrous sulfate, 0.00025% thiamin hydrochloride, 1.5% glucose, 1.5% casamino acid), to start aeration/agitation at 30°C. When the turbidity of the culture solution was approximately 500 Klett units, isopropyl- β -D-thiogalactopyranoside was added so that the final concentration was 12 mg/L, followed by another 4 hours of cultivation. After termination of cultivation, the culture solution was subjected to centrifugation to obtain approximately 660 g of wet cells, which were then frozen for storage at -80°C.

Example 29 (Acquisition of human apelin-36)

1,500 ml of 10 mM EDTA + 1 mM (p-amidophenyl)-methanesulfonyl fluoride hydrochloride (pH 6.0) solution was added to 550 g of the cells obtained in Example 28, followed by sonication (Branson Sonifier Model 450), followed by centrifugation (10,000 rpm, 60 min.). The supernatant was pooled, while the precipitate was subjected once again to the same procedure. The pooled supernatant was adjusted the pH to 6.0, then made to flow through an AF-Heparin Toyopearl 650M column (30 mm ID \times 500 mmL, Tosoh Corporation) equilibrated with 50 mM phosphate buffer solution (pH 6.0) for adsorption, followed by washing, then by elution on a step gradient of 0 to 100% B (B = 50 mM phosphate buffer solution + 2 M NaCl, pH 6.0), to obtain 530 ml of human apelin-36-CS23 fusion protein fractions.

This eluate was concentrated in a Pellicon Mini cassette (Millipore Corporation) while adding 0.1 M

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acetic acid, to obtain 0.1 M acetic acid solution of human apelin-36-CS23 fusion protein. After adding urea to this solution so that the final concentration was 6 M, 35 mg of 1-cyano-4-dimethylaminopyridinium (DMAP-CN) was added, and allowed to react for 15 minutes at room temperature. After termination of the reaction, the reaction solution was made to flow through a Sephadex G-25 column (46 mm ID × 600 mmL, Pharmacia) equilibrated with 10% acetic acid, the 10% acetic acid used for equilibration developed at a flow rate of 6 ml/min, and S-cyanylated human apelin-36-CS23 fusion protein fractions were obtained. This eluate was concentrated and desalted in a Pellicon Mini cassette (Millipore Corporation), to obtain the desalted solution of human apelin-36-CS23 fusion protein. After adding urea to this desalted solution so that the final concentration was 6 M, 1 N of caustic soda was added to bring the concentration to 0.06 N, and allowed to react for 15 minutes at 0°C. After termination of the reaction, the pH was adjusted to 6.0 with acetic acid, to obtain human apelin-36. This reaction solution was made to flow through an SP-5PW (21.5 mm ID × 150 mmL, Tosoh Corporation) equilibrated with 50 mM phosphate buffer solution (pH 6.5) containing 3 M urea, for adsorption, followed by washing, then by elution on a step gradient of 0 to 40% B (B = 50 mM phosphate buffer solution + 1 M NaCl + 3 M urea, pH 6.5), to obtain human apelin-36 fractions. These human apelin-36 fractions were made to flow through a C4P-50 (21.5 mm ID × 300 mmL, Showa Denko K.K.) equilibrated with 0.1% trifluoroacetic acid (TFA) for adsorption, followed by washing, then by elution on a step gradient of 15 to 30% B (B: 80% acetonitrile/0.1% TFA), whereupon the human apelin-36 fractions were pooled, then lyophilized, to obtain human apelin-36 lyophilized powder.

a) Amino acid composition analysis

The amino acid composition was determined using an amino acid analyzer (Hitachi L-8500A Amino Acid Analyzer).

- 5 The amino acid composition thus obtained matched the amino acid composition deduced from the base sequence of the DNA of human apelin-36 possessing N-terminal methionine (Table 15).

10 Table 15: Amino acid composition analysis

Amino acid	Number of residues per mole	Value deduced from human apelin-36 base sequence
Asx	1.0	1
Thr ¹⁾	0	0
Ser ¹⁾	1.9	2
Glx	3.0	3
Pro	5.7	6
Gly	5.7	6
Ala	0	0
Cys ²⁾	N.D.	0
Val	1.0	1
Met	2.0	1
Ile	0	0
Leu	2.0	2
Tyr	0	0
Phe	1.9	2
His	1.0	1
Lys	1.8	2
Arg	7.3	8
Trp	0.9	1

Acid hydrolysis (24-hour and 48-hour hydrolysis, at 110°C, with 6N hydrochloric acid - 4% thioglycolic acid).

- 15 1) Value extrapolated at 0 hours.

2) Not detected.

b) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Applied Biosystems Model 477A). Other than the human apelin-36 amino acid sequence thus obtained possessing a methionine residue on the N-terminal, said sequence matched the N-terminal amino acid sequence that was deduced from the DNA base sequence (Table 16).

Table 16: N-terminal amino acid sequence

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid deduced from human apelin-36 base sequence
1	Met (526)	
2	Leu (648)	Leu
3	Val (513)	Val
4	Gln (437)	Gln
5	Pro (463)	Pro
6	Arg (216)	Arg
7	Gly (232)	Gly
8	Ser (129)	Ser
9	Arg (129)	Arg
10	Asn (142)	Asn
11	Gly (185)	Gly
12	Pro (219)	Pro
13	Gly (202)	Gly
14	Pro (188)	Pro
15	Trp (88)	Trp
16	Gln (116)	Gln
17	Gly (120)	Gly
18	Gly (72)	Gly
19	Arg (56)	Arg

20	Arg (40)	Arg
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Analysis was conducted using 1 nmol.

1) Phenylthiohydantoin

c) C-terminal amino acid analysis

5 The C-terminal amino acid was analyzed using an amino acid analyzer (Hitachi L-8500A Amino Acid Analyzer) (Table 17).

Table 17: C-terminal amino acid analysis

10

Human apelin-36	C-terminal amino acid	Recovery rate (%)
	Phe	38.6

Phase hydrazinolysis (at 100°C for 6 hours)

15 The above analysis identified the human apelin-36 obtained in Example 29 as belonging to a molecular species that possesses methionine residue on the N-terminal (Met-apelin-36 (human)).

Example 30 (Measurement of biological activity)

20 The Met-apelin-36 (human) obtained in Example 29 was used to measure activity according to the method (Cytosensor) described in Example 6 of Patent Application Heisei 10-271645, whereupon said Met-apelin-36 (human) was verified to possess activity equivalent to that of synthetic human apelin-36.

25

Example 31 (Removal of N-terminal methionine residue)

30 After dissolving 4 mg of the Met-apelin-36 (human) obtained in Example 29 into 0.8 ml of 3 M urea solution, a mixture comprising 0.05 ml of 80 mM cupric sulfate, 0.046 g of glyoxylic acid, and 0.1 ml of pyridine was added, and allowed to react for 1 hour at 25°C. After termination of the reaction, the reaction solution was

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made to flow through a Sephadex G-25 column (10 mm ID × 250 mmL) equilibrated with 2.5 M urea + 10 mM phosphate buffer solution (pH 5.5), the solution used for equilibration developed at a flow rate of 0.5 ml/minute, and human apelin-36 fractions possessing diketone of the methionine residue were pooled. Subsequently, an equal volume of 2 M sodium formate, 4 M acetic acid, and 3 M urea solution was added to these fractions, followed by the addition of 3,4-diaminobenzoic acid so that the concentration was 40 mM, to be allowed to react for 3 days at 30°C. After termination of the reaction, the reaction solution was made to flow through a Sephadex G-25 column (25 mm ID × 600 mmL) equilibrated with 50 mM phosphate buffer solution (pH 6.0), the buffer solution used for equilibration developed at a flow rate of 4 ml/minute, and human apelin-36 fractions without N-terminal methionine residue were pooled. The pooled human apelin-36 fractions were adjusted the pH to 6.0, then adsorbed to a CM-5PW (7.5 mm ID × 75 mmL, Tosoh Corporation) equilibrated with 50 mM phosphate buffer solution + 0.1 M NaCl + 2.5 M urea (pH 5.0), followed by elution on a step gradient of 0 to 100% B (B = 50 mM borate buffer solution + 0.1 M NaCl + 2.5 M urea, pH 9.0) for 40 minutes at a flow rate of 0.8 ml/minute, whereupon human apelin-36 fractions were pooled. The human apelin-36 was then adsorbed to a C4P-50 (10 mm ID × 250 mmL, Showa Denko K.K.) equilibrated with 0.1% TFA, followed by elution on a step gradient of 15 to 30% B (B = 80% acetonitrile/0.1% TFA) for 40 minutes at a flow rate of 2 ml/minute. Upon pooling human apelin-36 fractions, said fractions were lyophilized, to yield human apelin-36.

a) Amino acid composition analysis

The amino acid composition was determined using an

amino acid analyzer (Hitachi L-8500A Amino Acid Analyzer).

The amino acid composition thus obtained matched the amino acid composition deduced from the base sequence of the DNA of hA10L (Table 18).

Table 18: Amino acid composition analysis

Amino acid	Number of residues per mole	Value deduced from the human apelin-36 base sequence
Asx	1.0	1
Thr ¹⁾	0	0
Ser ¹⁾	1.9	2
Glx	2.9	3
Pro	6.3	6
Gly	5.9	6
Ala	0	0
Cys ²⁾	N.D.	0
Val	1.0	1
Met	1.0	1
Ile	0	0
Leu	2.0	2
Tyr	0	0
Phe	1.9	2
His	1.0	1
Lys	1.9	2
Arg	7.6	8
Trp	0.9	1

Acid hydrolysis (24-hour and 48-hour hydrolysis, at 110°C, with 6N hydrochloric acid - 4% thioglycolic acid).

1) Value extrapolated at 0 hours.

2) Not detected.

b) N-terminal amino acid sequence analysis

The N-terminal amino acid sequence was determined using a gas phase protein sequencer (Applied Biosystems Model 477A). The amino acid sequence thus obtained matched the N-terminal amino acid sequence that was deduced from the base sequence of the DNA of the human apelin-36 obtained (Table 19).

Table 19: N-terminal amino acid sequence

Residue No.	PTH ¹⁾ Amino acid detected (pmol)	Amino acid deduced from human apelin-36 base sequence
1	Leu (475)	Leu
2	Val (845)	Val
3	Gln (365)	Gln
4	Pro (563)	Pro
5	Arg (425)	Arg
6	Gly (424)	Gly
7	Ser 138)	Ser
8	Arg (423)	Arg
9	Asn (245)	Asn
10	Gly (290)	Gly
11	Pro (197)	Pro
12	Gly (234)	Gly
13	Pro (197)	Pro
14	Trp (101)	Trp
15	Gln (76)	Gln
16	Gly (84)	Gly
17	Gly (130)	Gly
18	Arg (79)	Arg
19	Arg (116)	Arg
20	Lys (43)	Lys

10 Analysis was conducted using 1 nmol.

1) Phenylthiohydantoin

c) C-terminal amino acid analysis

The C-terminal amino acid was analyzed using an amino acid analyzer (Hitachi L-8500A Amino Acid Analyzer) (Table 20).

5

Table 20: C-terminal amino acid analysis

Human apelin-36	C-terminal amino acid	Recovery rate (%)
	Phe	86.6

Phase hydrazinolysis (at 100°C for 6 hours)

10

Example 32 (Measurement of biological activity)

The human apelin-36 obtained in Example 31 was used to measure activity according to the method (Cytosensor) described in Example 6 of Patent

15

Application Heisei 10-271646, whereupon said human apelin-36 was verified to possess activity equivalent to that of synthetic human apelin-36.

INDUSTRIAL APPLICABILITY

20

The present invention makes possible the selective, specific and efficient removal of methionine residue from peptides, proteins, or the salts thereof which possess optionally oxidized N-terminal methionine residue, and makes possible the efficient production of

25

peptides, proteins, or the salts thereof which do not possess optionally oxidized N-terminal methionine residue. Additionally, according to the methods of the present invention, the chemical removal of the N-terminal methionine residue is possible under mild

30

conditions regardless of the type of peptide or protein, thereby making possible the industrially advantageous manufacture of peptides or proteins possessing wild-type amino acid sequences using, as raw material,

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genetically engineered peptides, proteins, or the salts thereof which possess methionine residue.

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CLAIMS

1. A method for removing the diketone of the optionally oxidized N-terminal methionine residue, that is
5 characterized by having a peptide or a salt thereof which possesses a diketone of the optionally oxidized N-terminal methionine residue react with 3,4-diaminobenzoic acid or a salt thereof in the presence of acetic acid and sodium formate, formic acid and
10 sodium formate, or formic acid and sodium acetate.
2. The method described in Claim 1 wherein the peptide or the salt thereof which possesses a diketone of the optionally oxidized N-terminal methionine residue is a peptide or a salt thereof which is obtained by having a
15 peptide or a salt thereof which possesses optionally oxidized N-terminal methionine residue react with an α -diketone.
3. The method described in Claim 2 wherein the peptide which possesses optionally oxidized N-terminal
20 methionine residue is a peptide which has been manufactured by genetic engineering technology.
4. The method described in Claim 1 wherein the peptide is (i) a growth hormone, (ii) beta-cellulin, (iii) interleukin-2, (iv) neurotrophin-3, or (v) apelin.
- 25 5. The method described in Claim 1 wherein the peptide is a growth hormone.
6. The method described in Claim 1 that is characterized by the acetic acid and sodium formate, formic acid and sodium formate, or formic acid and
30 sodium acetate being used as a buffer solution of approximately 0.1 to 8 mol/L, with a pH of approximately 2 to 9.
7. A method for removing the diketone of the optionally oxidized N-terminal methionine residue that is
35 characterized by having a peptide or a salt thereof

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which possesses a diketone of the optionally oxidized N-terminal methionine residue react with 3,4-diaminobenzoic acid or a salt thereof in the presence of acetic acid and sodium formate.

5 8. A method for the manufacture of a peptide or a salt thereof which does not possess optionally oxidized N-terminal methionine residue characterized by having a peptide or a salt thereof which possesses a diketone of the optionally oxidized N-terminal methionine residue
10 react with 3,4-diaminobenzoic acid or a salt thereof in the presence of acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate.

9. The method of manufacture described in Claim 8
15 wherein the peptide or the salt thereof which possesses a diketone of the optionally oxidized N-terminal methionine residue is a peptide or salt thereof obtained by having a peptide or salt thereof which possesses optionally oxidized N-terminal methionine
20 residue react with an α -diketone.

10. The method of manufacture described in Claim 8 that is characterized by the acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate being used as a buffer solution of
25 approximately 0.1 to 8 mol/L, with a pH of approximately 2 to 9.

11. A method for manufacturing a peptide or a salt thereof which does not possess N-terminal methionine residue characterized by having a peptide or salt
30 thereof which possesses a diketone of the N-terminal methionine residue react with 3,4-diaminobenzoic acid or a salt thereof in the presence of acetic acid and sodium formate.

12. A method for manufacturing human growth hormone or
35 a salt thereof which does not possess N-terminal

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methionine residue characterized by having a genetically engineered peptide or salt thereof which possesses optionally oxidized N-terminal methionine residue react with glyoxylic acid or a salt thereof in the presence of cupric sulfate and pyridine, then with 3,4-diaminobenzoic acid or a salt thereof in the presence of acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate.

13. The use of (i) acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or a salt thereof, for the purpose of removing the methionine residue from a peptide or a salt thereof which possesses optionally oxidized N-terminal methionine residue.

14. The use of (i) acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or a salt thereof, for the purpose of removing the diketone of the methionine residue from a peptide or a salt thereof which possesses a diketone of the optionally oxidized N-terminal methionine residue.

15. The use of (i) acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or a salt thereof, for the purpose of manufacturing a peptide or a salt thereof which does not possess optionally oxidized N-terminal methionine residue from a peptide or a salt thereof which possesses optionally oxidized N-terminal methionine residue.

16. The use of (i) acetic acid and sodium formate, formic acid and sodium formate, or formic acid and sodium acetate, and (ii) 3,4-diaminobenzoic acid or a salt thereof, for the purpose of manufacturing a peptide or a salt thereof which does not possess a

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diketone of optionally oxidized N-terminal methionine residue from a peptide or a salt thereof which possesses a diketone of optionally oxidized N-terminal methionine residue.

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Fig.1

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(kDa)

97.4

66.2

45.0

31.0

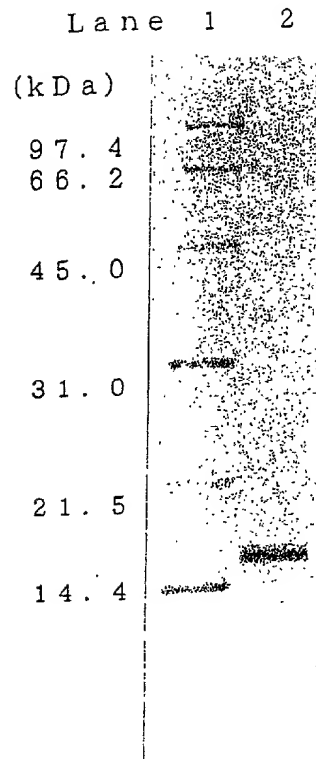
21.5

14.4



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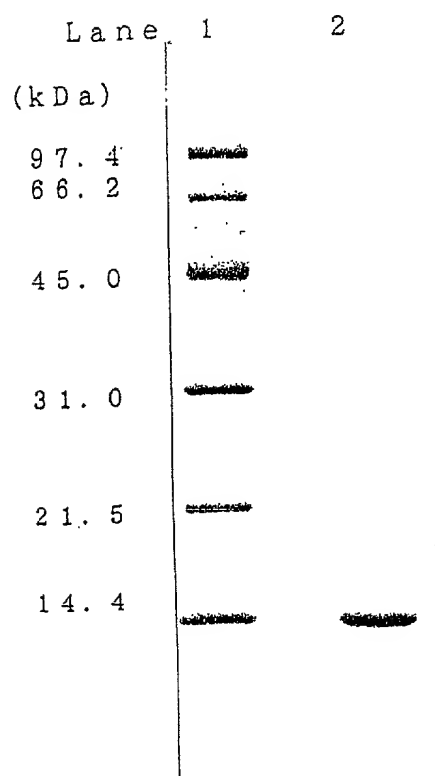
Fig.2



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Fig.3

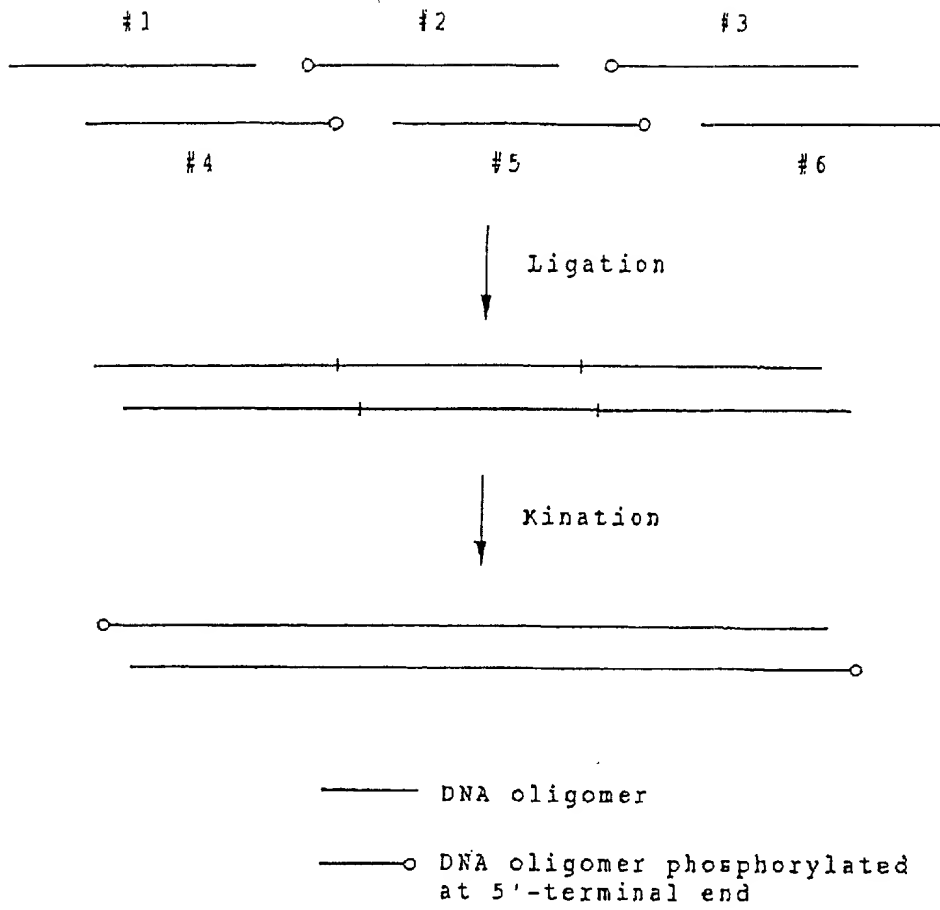


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Fig.4

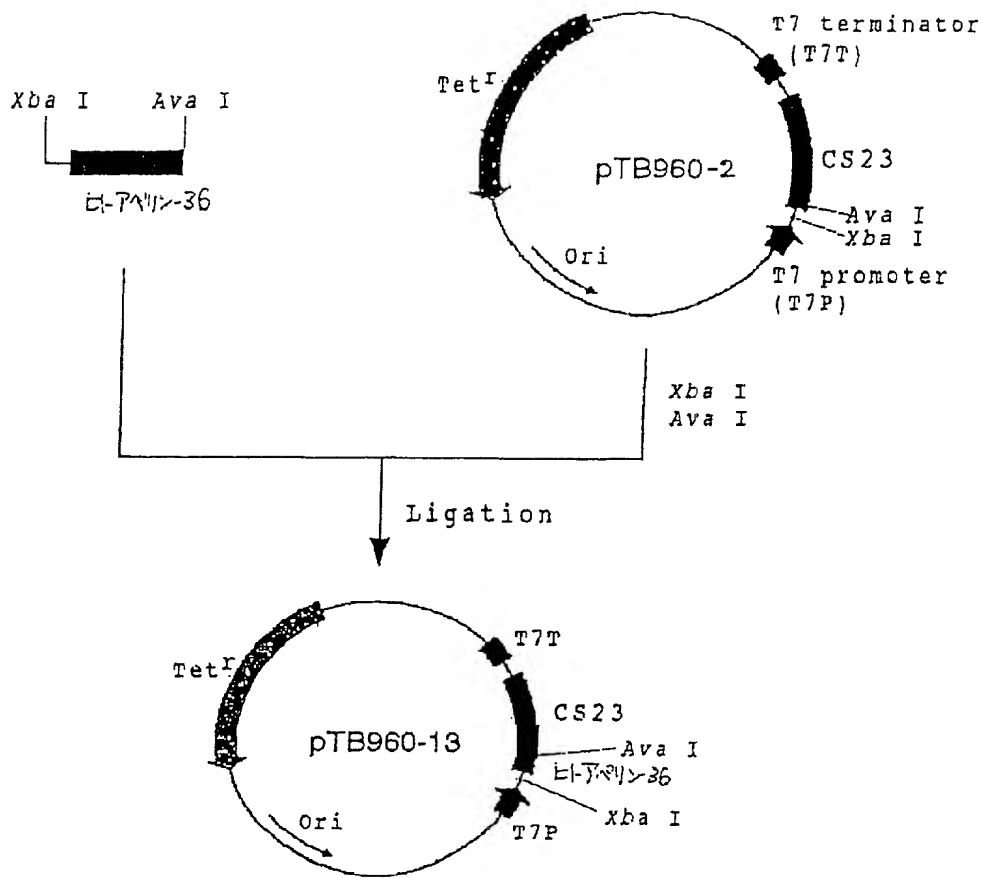
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#3 5'GTCAACGTCGCGTCTGTCTCATAAAGGTCCGATGCCGTTTGGCC
#4 5'GACCATTACGAGAACCAACGCGGTGAAACCAGCATATGATATCTCCTTT
#5 5'GGACGTTGACGACGAAATTACGACGACCACTTGCCATGGACCCG
#6 5'TCGGGGCAAAACGGCATCGGACCTTTATGAGACAGACGC

Fig.5



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Fig.6



DECLARATION AND POWER OF ATTORNEY FOR U.S. PATENT APPLICATION

(X) Original () Supplemental () Substitute (X) PCT () DESIGN

As a below named inventor, I hereby declare that: my residence, post office address and citizenship are as stated below next to my name; that I verily believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Title: Method for Removing N-terminal Methionine

of which is described and claimed in:

() the attached specification, or
 () the specification in application Serial No. _____, filed _____, and with amendments through _____, or
 (X) the specification in International Application No. PCT/JP99/05456, filed October 4, 1999, and as amended on (if applicable).

I hereby state that I have reviewed and understand the content of the above-identified specification, including the claims, as amended by any amendment(s) referred to above.

I acknowledge my duty to disclose to the Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim priority benefits under Title 35, United States Code, §119 (and §172 if this application is for a Design) of any application(s) for patent or inventor's certificate listed below and have also identified below any application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:


COUNTRY	APPLICATION NO.	DATE OF FILING	PRIORITY CLAIMED
JP	282476/1998	October 5, 1998	Yes

I hereby claim the benefit under Title 35, United States Code §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	U.S. FILING DATE	STATUS: PATENTED, PENDING, ABANDONED

And I hereby appoint Michael R. Davis, Reg. No. 25,134; Matthew M. Jacob, Reg. No. 25,154; Warren M. Cheek, Jr., Reg. No. 33,367; Nils Pedersen, Reg. No. 33,145; Charles R. Watts, Reg. No. 33,142; and Michael S. Huppert, Reg. No. 40,268, who together constitute the firm of WENDEROTH, LIND & PONACK, L.L.P., as well as any other attorneys and agents associated with Customer No. 000513, to prosecute this application and to transact all business in the U.S. Patent and Trademark Office connected therewith.

I hereby authorize the U.S. attorneys and agents named herein to accept and follow instructions from Abe, Ikubo & Katayama as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. attorneys and myself. In the event of a change in the persons from whom instructions may be taken, the U.S. attorneys named herein will be so notified by me.

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I further declare that all statements made herein of my own knowledge are true, and that all statements on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

1st Inventor Osamu Nishimura Date March 30, 2001
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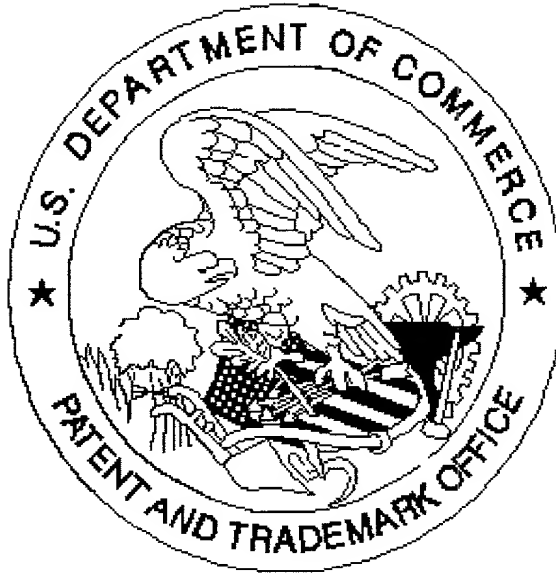
The above application may be more particularly identified as follows:

U.S. Application Serial No. _____ Filing Date _____

Applicant Reference Number _____ Atty Docket No. _____

Title of Invention _____

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